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# Role of Cytochrome *c* Heme Lyase in Mitochondrial Import and Accumulation of Cytochrome *c* in *Saccharomyces cerevisiae*

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**Heme is covalently attached to cytochrome *c* by the enzyme cytochrome *c* heme lyase. To test whether heme attachment is required for import of cytochrome *c* into mitochondria in vivo, antibodies to cytochrome *c* have been used to assay the distributions of apo- and holocytochromes *c* in the cytoplasm and mitochondria from various strains of the yeast *Saccharomyces cerevisiae*. Strains lacking heme lyase accumulate apocytochrome *c* in the cytoplasm. Similar cytoplasmic accumulation is observed for an altered apocytochrome *c* in which serine residues were substituted for the two cysteine residues that normally serve as sites of heme attachment, even in the presence of normal levels of heme lyase. However, detectable amounts of this altered apocytochrome *c* are also found inside mitochondria. The level of internalized altered apocytochrome *c* is decreased in a strain that completely lacks heme lyase and is greatly increased in a strain that overexpresses heme lyase. Antibodies recognizing heme lyase were used to demonstrate that the enzyme is found on the outer surface of the inner mitochondrial membrane and is not enriched at sites of contact between the inner and outer mitochondrial membranes. These results suggest that apocytochrome *c* is transported across the outer mitochondrial membrane by a freely reversible process, binds to heme lyase in the intermembrane space, and is then trapped inside mitochondria by an irreversible conversion to holocytochrome *c* accompanied by folding to the native conformation. Altered apocytochrome *c* lacking the ability to have heme covalently attached accumulates in mitochondria only to the extent that it remains bound to heme lyase.**

Like most mitochondrial proteins, cytochrome *c* is synthesized in the cytoplasm and then translocated into mitochondria. However, the pathway of cytochrome *c* import to its final location on the outer surface of the inner mitochondrial membrane differs from that of most other imported proteins, even those targeted to the same mitochondrial compartment. Cytochrome *c* import does not depend on a membrane potential across the inner mitochondrial membrane, does not involve import into and export from the mitochondrial matrix, does not involve receptors on the external mitochondrial surface, and does not require ATP (18, 29).

Mature cytochrome *c* contains a protoheme group covalently attached via thioether linkages to two cysteine residues of the protein. Attachment of heme to the imported precursor, apocytochrome *c*, is catalyzed by the enzyme cytochrome *c* heme lyase (also called holocytochrome-*c* synthase; EC 4.4.1.17) (9). Based on experiments using isolated mitochondria, covalent attachment of heme appears to be coupled to import of the protein into mitochondria. An excess of deuterohemin, an analog of protoheme that cannot be attached to apocytochrome *c*, blocks import of precursor into isolated mitochondria (19). Isolated mitochondria lacking cytochrome *c* heme lyase do not import apocytochrome *c* (10, 28). Altered forms of apocytochrome *c* containing single amino acid substitutions or chemical modifications at residues at the site of heme attachment to apocytochrome *c* exhibit reduced import into isolated mitochondria (10). However, recently Hakvoort et al. (15) have suggested that cytochrome *c* import can occur independently of heme attachment.

The gene encoding cytochrome *c* heme lyase has been cloned from both the yeast *Saccharomyces cerevisiae* (9) and *Neurospora crassa* (7). Although heme attachment to cytochrome *c* has been shown to require NADH or other agents to keep the heme reduced (31), little is known about its mode of action or the coupling of heme attachment to import. The submitochondrial localization of the enzyme is of particular interest because of its role in import. Heme lyase activity has been found to be associated with mitochondria (4) and, more particularly, the mitochondrial intermembrane space (13, 29).

We report here the results of investigations, carried out in vivo, of the import of cytochrome *c* into the mitochondria of an isogenic series of yeast strains. We find that apocytochrome *c* can accumulate in mitochondria under conditions in which heme cannot be attached. However, the presence of cytochrome *c* heme lyase is required for accumulation of apocytochrome *c* inside mitochondria, even when the enzyme is not acting catalytically. Mitochondria expressing high levels of heme lyase accumulate apocytochrome *c* with high efficiency. We have also raised antibodies that specifically recognize heme lyase and used these to demonstrate that the enzyme is tightly associated with the outer surface of the inner mitochondrial membrane and not enriched at sites of contact between the two mitochondrial membranes. The results are consistent with a model in which apocytochrome *c* reversibly crosses the outer mitochondrial membrane, diffuses to the inner membrane, then is trapped in the intermembrane space, either by heme attachment or by binding to heme lyase.

## MATERIALS AND METHODS

**Yeast strains and genetic nomenclature.** Relevant genotypes of the yeast strains used are shown in Table 1. *CYC1* and *CYC7* are the structural genes encoding iso-1-cy-

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TABLE 1. Relevant genotypes, holocytochrome *c* contents, and mitochondrial cytochrome *c* contents of yeast strains

| Strain | Relevant genotype[a] | Approx holocytochrome *c* level (%)[b] | Cytochrome *c* in mitochondria (%)[c] |
|---|---|---|---|
| B-7908 | *cyc1-783lacZ CYC7-H3 CYC3*$^+$ | 150 | 95 |
| B-7911 | *cyc1-783::lacZ cyc7-H3-67 CYC3*$^+$ | <5 | 3 |
| B-8076 | *cyc1-783::lacZ CYC7-H3 cyc3-Δ::LEU2* | <5 | 0.5 |
| B-8077 | *cyc1-783::lacZ cyc7-H3-67 cyc3-Δ::LEU2* | <5 | 0.5 |
| B-8118 | *cyc1-783::lacZ cyc7-H3-67 CYC3*$^+$ [pAB790] | <5 | 40 |
| B-614 | *CYC1*$^+$ *CYC7*$^+$ *cyc3-10* | 5 | ND |
| B-619 | *CYC1*$^+$ *CYC7*$^+$ *cyc3-15* | 5 | ND |
| D311-3A | *CYC1*$^+$ *CYC7*$^+$ *CYC3*$^+$ | 100 | ND |
| B-7034 | *CYC1*$^+$ *CYC7*$^+$ *CYC3*$^+$ | 100 | ND |
| B-6868 | *CYC1*$^+$ *CYC7*$^+$ *CYC3*$^+$ [pAA268] | 100 | ND |

[a] The *cyc1-783::lacZ* allele is a deletion of the *CYC1* locus causing a complete deficiency of iso-1-cytochrome *c*. The *cyc7-H3-67* allele encodes iso-2-cytochrome *c* that has been altered by substituting serine residues for cysteine residues 23 and 26 (see Materials and Methods). The genotype *CYC3*$^+$ [pAB790] indicates that the strain contains the *CYC3* gene under control of the actin promoter on the TRP1-containing multicopy plasmid pAB790 (see Materials and Methods). The genotype *CYC3*$^+$ [pAB268] indicates that the strain contains the *CYC3* gene under control of the actin promoter on the *URA3*-containing multicopy plasmid pAB268 (9).

[b] Determined by low-temperature spectroscopy of whole cells (40).

[c] Mitochondrial cytochrome *c* contents for strains B-7911, B-8076, and B-8077, containing low levels of mitochondrial cytochrome *c*, were calculated from the comparisons of the cytochrome *c* concentrations per milligram of total protein of crude cell extracts and mitochondria, measured at various dilutions by immunoblotting. For purposes of this calculation, mitochondria were assumed to constitute 5% of total cell protein. Mitochondrial cytochrome *c* contents for strains B-7908 and B-8118, containing high levels of mitochondrial cytochrome *c*, were calculated from comparisons of the relative cytochrome *c* concentrations per milligram of protein of the crude extract and postmitochondrial fractions, also measured at various dilutions on immunoblots. The percent depletion of cytochrome *c* in the postmitochondrial fraction compared with the crude extract was assumed to equal the percent cytochrome *c* in mitochondria. ND, not determined.

tochrome *c* and iso-2-cytochrome *c*, the two isozymes of cytochrome *c* in *S. cerevisiae*. The *cyc1-783::lacZ* allele contains a replacement of the coding region of the *CYC1* locus by the *Escherichia coli lacZ* gene (20). *CYC7-H1* and *CYC7-H3* are alleles of *CYC7* that produce abnormally high levels of iso-2-cytochrome *c* (11). *CYC3* encodes the yeast cytochrome *c* heme lyase (9, 10).

**Construction of strains.** To create the *cyc7-H3-67* allele, containing serine residues substituted for the cysteines that serve as sites of heme attachment to iso-2-cytochrome *c*, the coding region of *CYC7-H3* was altered by site-directed mutagenesis. A 4.6-kb *Hin*dIII-*Hin*dIII fragment containing the *CYC7-H3* gene, originally from plasmid pAB38 (26), was ligated into the *Hin*dIII site of pEMBL-YI32, containing the *URA3*$^+$ gene (2). A mutagenic oligonucleotide of the sequence TAAAACGAGGTCTCAGCAGTCTCATACAAT AG was used to change the codons for the two cysteine residues (TGT) to codons for serine (TCT) by the method of Kunkel et al. (21) essentially as described previously (5). Both the mutagenized (pAB599) and normal (pAB595) versions of the plasmid were integrated at the chromosomal *CYC7* locus of strain B-6748 (*MAT***a** *cyc1-783::lacZ cyc7-Δ::CYH2 ura3-52 his3-Δ1 leu2-3,112 trp1-289 cyh2*) to create strains B-7911 (*cyc7-H3-67*) and B-7908 (*CYC7-H3*), respectively, using techniques described previously (20). Genomic Southern blots demonstrated that in each case a single copy of *CYC7-H3* recombined 5′ to the preexisting *cyc7-Δ::CYH2* locus. The sequence of the *cyc7-H3-67* allele was verified by double-stranded sequencing of a 1-kb fragment of DNA amplified by the polymerase chain reaction, using primers 50 bp 5′ to the breakpoint of the *CYC7-H3* deletion and 320 bp 3′ to the termination codon of *CYC7*.

The *CYC3* gene was deleted from strains B-7908 and B-7911 by using plasmid pAB753, in which the coding region has been replaced by *LEU2*. This plasmid was, in turn, derived from plasmid pAB609, containing a replacement of the *CYC3* coding region by the *URA3* gene. To create pAB609, a 4.5-kb *Hin*dIII-*Eco*RI genomic fragment containing an in vitro-mutagenized version of *CYC3* with an *Eco*RI site introduced just 5′ to the *CYC3* coding region (9) was digested completely with *Sal*I and partially with *Eco*RI to remove a 1.1-kb fragment containing the coding region. This *CYC3* fragment was replaced with the *URA3* gene on a 1.1-kb *Eco*RI-*Sal*I fragment to create pAB609. Following digestion of pAB609 with *Sal*I and *Hin*dIII, a 6-kb fragment from this plasmid was ligated to a 2.9-kb *Sal*I-*Hin*dIII fragment from YIp32, containing the *LEU2* gene, to create pAB753. Strains B-7908 and B-7911 were transformed with *Ava*I-digested pAB753, using a lithium acetate procedure (27), to produce strains B-8076 and B-8077, respectively. *LEU2*$^+$ colonies were picked, genomic DNA was isolated, and the disruption was verified by using the polymerase chain reaction, monitoring the 1.1-kb increase in the size of the disrupted, compared with the normal, locus.

Plasmid pAB790, a multicopy plasmid containing *TRP1* and the *CYC3* gene fused to the actin promoter, was derived from plasmid pAA268, containing *URA3* instead of TRP1 (9). A 1.5-kb *Bam*HI-*Sal*I fragment from pAA268 containing the actin-*CYC3* fusion was ligated to a 5.4-kb *TRP1*-containing *Bam*HI-*Sal*I fragment from YRp7.

**Subcellular fractionation.** Subcellular fractions were prepared as described by Daum et al. (6), with the following exceptions. (i) For the experiments shown in Fig. 1 and 2, cells were lysed in 0.5 M mannitol–0.1 M KCl–20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH–1 mM EDTA–0.1% bovine serum albumin–1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4. The crude extract fraction was the supernatant from the first low-speed spin (1,500 × *g*, 5 min) to remove unbroken cells and cell membranes. After centrifugation at 10,000 × *g* for 10 min to separate the mitochondria from the postmitochondrial supernatant, the mitochondria were recentrifuged once in the same buffer used for lysis and then centrifuged again in KMEH buffer (0.5 M mannitol, 0.1 M KCl, 20 mM HEPES-KOH, 1 mM EDTA, pH 7.4). (ii) The broken-cell fractions in Fig. 3 were obtained as described by Duell et al. (8) from spheroplasts prepared with Glusulase and then lysed in 0.25 M sucrose–50 mM potassium phosphate–1 mM EDTA, pH

6.8. (iii) For experiments with mitoplasts shown in Fig. 4, 20 mM morpholinepropanesulfonic acid (MOPS; pH 7.4) was substituted for HEPES in all buffers. (iv) For the submitochondrial fractionation shown in Fig. 5, cells were lysed in 0.6 M mannitol–20 mM HEPES-KOH–10 mM EDTA–0.1% bovine serum albumin–1 mM PMSF, pH 7.4. Subsequent steps in the isolation contained no EDTA. (v) For the experiments shown in Fig. 1 and 3, strains were cultured in YPD. For the experiments shown in Fig. 2, strains were cultured in SD containing 0.1% Casamino Acids plus histidine and leucine (strain B-8118) or histidine, leucine, and tryptophan (strain B-7911) (39).

Proteinase K digestions were carried out at 1 to 2 mg of mitochondrial protein and 0.35 mg of enzyme per ml for 30 min at 0°C, followed by inhibition with PMSF as described previously (10). Where indicated, Triton X-100 was present at 0.5%. In preparation for storage or electrophoresis, all cell fractions were diluted 1:3 in fourfold-concentrated loading buffer for sodium dodecyl sulfate (SDS)-gel electrophoresis containing 4 mM PMSF and then immediately heated in a boiling water bath for 5 min.

Mitoplasts were prepared by osmotic disruption of the outer mitochondrial membrane (6). Release of mitochondrial markers was assayed following centrifugation of mitoplasts at 30,000 × *g* for 40 min at 4°C. The inner and outer membranes were separated by osmotic shrinking and swelling, followed by sonication and sucrose gradient fractionation essentially as described by Pon et al. (35). Sucrose gradient fractionation of mitochondrial membranes was found to be quite sensitive to the conditions used for sonication. Individual batches of swollen and shrunken mitochondria containing 35 mg of total protein in 13 ml of buffer were subjected to sonication for a total of 5 min in 30-s intervals interspersed with 3 min of cooling. The 0.5-in. (ca. 1.3-cm) probe of a Heat Systems Ultrasonics Inc. W-140 instrument was used at 70% of full power. Intact mitochondria and large fragments were removed by centrifugation at 27,000 × *g* for 15 min. Submitochondrial vesicles were collected by centrifugation at 185,000 × *g* for 2 h.

The intermembrane space marker cytochrome $b_2$ (1), the inner membrane marker cytochrome *c* oxidase (24), and the matrix marker fumarase (36) were assayed as described except that assays for cytochrome $b_2$ and fumarase were carried out in the presence of 0.1% Triton X-100, except as noted. Protein concentrations were determined by using a modified Lowry procedure (34). Cytoplasmic contamination of mitochondria was assayed by using the enzyme α-glucosidase (16). The activity of this enzyme was too low in cells grown on SD medium to allow precise estimation of the level of cytoplasmic contamination of the mitochondria from either strain used for the experiment shown in Fig. 2; however, the level of apocytochrome *c* detected inside the mitochondria of strain B-7911 in this experiment was comparable to that obtained under other growth conditions.

**Immunologic procedures.** The anti-cytochrome *c* antibodies used for immunoblotting have been described previously (11). Antibodies recognizing cytochrome *c* heme lyase were prepared by using synthetic peptides (purchased from Biosearch) corresponding to two regions of the predicted amino acid sequence of the *CYC3* gene product, the COOH-terminal 11 residues (ISGPSSSSSAP) and the internal sequence encompassing amino acid positions 219 to 229 (DFYGGPDDENG). This second peptide was synthesized with an amide in place of the carboxyl group of the terminal glycine. Peptides were conjugated to keyhole limpet hemocyanin (Calbiochem) essentially as described previously (3). Mixtures of conjugated and free peptide were used to immunize rabbits. Antibodies recognizing yeast mitochondrial outer membrane porin were a generous gift of the laboratory of Gottfried Schatz.

Immunoblotting of SDS-polyacrylamide gels was performed as described previously (11). Primary antibodies were diluted 1:300 in 5% newborn calf serum in 140 mM NaCl–10 mM sodium phosphate pH 7.4. Peroxidase-conjugated goat anti-rabbit secondary antibodies (Bio-Rad Laboratories) were used at 1:1,500. Ratios of antigen concentrations in different subcellular fractions were measured by comparing dilutions of the different fractions on the same immunoblot and identifying the dilutions that gave equal intensities of staining.

## RESULTS

**Role of heme attachment in the subcellular localization of cytochrome *c*.** Import of cytochrome *c* into the mitochondria of living cells was investigated by isolating subcellular fractions from an isogenic series of yeast strains and immunoblotting the fractions with cytochrome *c*-specific antibodies. The results are summarized in Table 1. These experiments could not have been carried out in strains bearing normal structural genes for the two isozymes of cytochrome *c* because of the intracellular instability of the apo form of iso-1-cytochrome *c*. Iso-1-cytochrome *c* constitutes 95% of cellular cytochrome *c* under normal laboratory growth conditions, with iso-2-cytochrome *c* making up the remaining 5%. However, if it is not imported into mitochondria and converted to holocytochrome *c*, apo-iso-1-cytochrome *c* has a half-life on the order of a few minutes and cannot be detected by immunoblotting. In contrast, apo-iso-2-cytochrome *c* is stable for at least 1 h under similar conditions (11). Thus, we used strains that completely lacked iso-1-cytochrome *c*, because of deletions of the *CYC1* gene, but expressed high levels of iso-2-cytochrome *c* as a result of a mutation in the 5′ untranslated region of *CYC7*. This type of strain had previously been shown to produce high levels of apo-iso-2-cytochrome *c* (11, 25).

A subcellular fractionation of B-7908, a *CYC3*$^+$ *CYC7-H3* strain that expresses high levels of holo-iso-2-cytochrome *c*, is shown in Fig. 1A. As expected, most of the immunoreactive cytochrome *c* was found in the mitochondria (Table 1; Fig. 1A, lane 4) in a compartment that was inaccessible to digestion by external proteases (lane 5) unless detergent was added to disrupt the membranes (lane 6). Cytochrome *c* was present only at low levels in the cytoplasm (lane 3), compared with whole spheroplasts (lane 1) or crude extract (lane 2). A thin band of cross-reacting material is visible just above the band corresponding to cytochrome *c* in lanes 1 to 3.

Cross-contamination of the cytoplasmic fraction by contents of the mitochondrial intermembrane space was estimated from activity of cytochrome $b_2$, assayed in the presence of 0.1% Triton X-100. About 10% of the total cellular activity of this enzyme was found in the cytoplasmic fractions, leading to specific activities more than 300-fold lower in these fractions than in mitochondria. This level of contamination, combined with the 10-fold-greater amount of cytoplasmic protein loaded on gels, compared with mitochondrial protein, explains the low level of cytochrome *c* detected in the cytoplasmic fraction of B-7908 (Fig. 1A, lane 3).

After subcellular fractionation, cytochrome *c* was found mainly in the cytoplasm of strain B-7911 (*CYC3*$^+$ *cyc7-H3-67*), which is isogenic with B-7908 except that the two

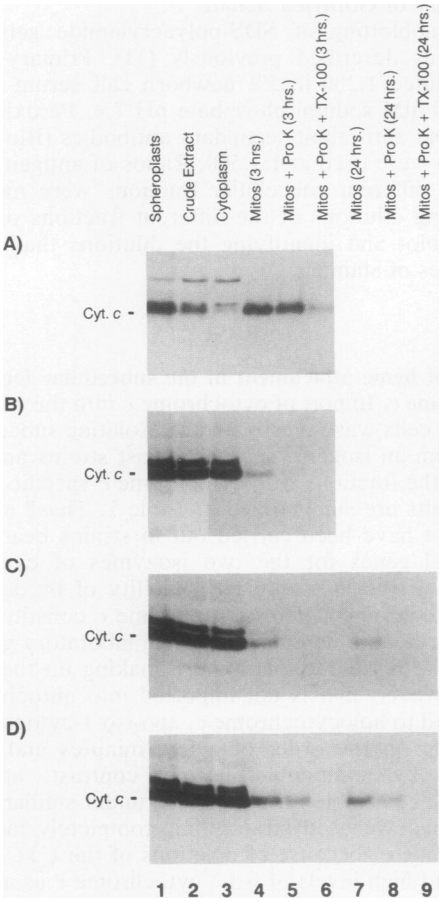


FIG. 1. Subcellular distributions of cytochrome *c* in various yeast strains. Yeast strains were grown, harvested, and fractionated as described in Materials and Methods. Samples of each fraction were electrophoresed on a 10% acrylamide-SDS gel (38), transferred to nitrocellulose, and probed with anti-cytochrome *c* antibodies followed by horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G. (A) Fractionation of strain B-7908 (*CYC7-H3 CYC3*$^+$). A total of 100 μg of protein was loaded on lanes 1 to 3, and 10 μg was loaded on lanes 4 to 6. (B) Fractionation of strain B-8077 (*cyc7-H3-67 cyc3*-Δ) expressing iso-2-cytochrome *c* with serine residues replacing the two cysteines residues, 23 and 26, involved in heme attachment and a deletion of the gene encoding heme lyase. A total of 200 μg of protein was loaded on lanes 1 to 3, and 20 μg was loaded on lanes 4 to 6. (C) Fractionation of strain B-8076 (*CYC7-H3-cyc3*-Δ) expressing the normal iso-2-cytochrome *c* sequence and containing a deletion of heme lyase. A total of 200 μg of protein was loaded on lanes 1 to 3, and 20 μg was loaded on lanes 4 to 9. (D) Fractionation of strain B-7911 (*cyc7-H3-67 CYC3*$^+$) expressing iso-2-cytochrome *c* with the serine-to-cysteine replacements. Sample loadings were as for panel C. Mitochondria were stored on ice for the indicated times following cell breakage, diluted fivefold in KMEH buffer, and treated as follows: lanes 4 and 7 (Mitos), mitochondria were incubated at 0°C for 30 min; lanes 5 and 8 (Mitos +, Pro K), mitochondria were treated with proteinase K (0.35 mg/ml) at 0°C for 30 min; lanes 6 and 9 (Mitos + Pro, K + TX-100), mitochondria were treated with proteinase K as for lane 5 but in the presence of 0.5% Triton X-100. The migration of iso-2-cytochrome *c* is indicated at the left.

cysteine residues that form the thioether linkage to heme in the normal protein were changed to serine residues by site-directed mutagenesis (Fig. 1D). A predominantly cytoplasmic localization of immunoreactive cytochrome *c* was also seen in the heme lyase deletion strains B-8076 (*cyc3*-Δ *CYC7-H3*) and B-8077 (*cyc3*-Δ *cyc7-H3-67*) (Fig. 1B and C; Table 1). Thus, blockage of heme attachment, either by alteration of the heme attachment site of cytochrome *c* or by mutation of the heme lyase, leads to accumulation of apocytochrome *c* in the cytoplasm and a significant decrease in levels of cytochrome *c* in mitochondria.

Although strain B-7911 (*CYC3*$^+$ *cyc7-H3-67*) contained most of its apocytochrome *c* in the cytoplasm, a low level of cytochrome *c* was detectable in the mitochondrial fraction (Fig. 1D, lanes 4 and 5; note that approximately 1/10 the amount of total protein was loaded on the lanes 4 to 6, containing the mitochondrial fractions, compared with lanes 1 to 3, containing cytoplasm and whole-cell extracts). By comparing immunoblots of dilutions of the mitochondrial and cytoplasmic fractions, the cytochrome *c* concentration (per milligram of total protein) in protease-treated mitochondrial fraction from this strain was estimated to be 60% of the cytoplasmic concentration (Table 1). Cytoplasmic contamination of the mitochondrial fraction is less than 10%, based on comparisons of the α-glucosidase activity per milligram of protein in the fractions. Furthermore, much of the cytochrome *c* in this fraction is resistant to protease, as would be expected for a protein inside mitochondria. Thus, the altered *cyc7-H3-67* protein, which should be incapable of covalently binding heme, must be imported at a low level. Making the assumption that mitochondria constitute 5% of cell protein (based on comparisons of mitochondrial and cytoplasmic cytochrome $b_2$ activity and cytochrome *c* levels in Fig. 1A, lanes 1 and 4), the fraction of the altered protein found inside mitochondria can be estimated at about 3% of the total cellular pool.

The mitochondrial fractions of the heme lyase-deficient strains B-8076 and B-8077 (Fig. 1B and C) contained considerably less cytochrome *c* than does the *CYC3*$^+$ *cyc7-H3-67* strain B-7911. Following proteinase K digestion, the cytochrome *c* concentration in mitochondria of the *cyc3* strains was about 10% of the cytoplasmic concentration (amount of cytochrome *c* per milligram of total protein), implying that only 0.5% of the cellular cytochrome *c* is inside mitochondria. This low level is comparable to cytoplasmic contamination of mitochondria; thus, we cannot be sure that any cytochrome *c* is, in fact, imported into these mitochondria.

The lower level of import observed in strains that could not attach heme because of a deletion of heme lyase, compared with *CYC3*$^+$ strains that could not attach heme because of mutation of the cysteine residues involved in thioether formation, suggested that an interaction between heme lyase and apocytochrome *c*, even in the absence of productive catalysis of heme attachment, might play a role in mitochondrial cytochrome *c* accumulation. To test this possibility, we compared import of the *cyc7-H3-67* altered apocytochrome *c* in two strains that differed only in the presence or absence of a multicopy plasmid, pAB790, causing expression of high levels of heme lyase. As seen in Fig. 2 and Table 1, increased expression of heme lyase in strain B-8118, containing the multicopy plasmid, led to a dramatic increase in import of apocytochrome *c*. Proteinase K-treated mitochondria from this strain contained about 40% of the total cellular cytochrome *c*, meaning that the mitochondrial concentration was more than 10 times the cytoplasmic concentration per milligram of total protein. At least a

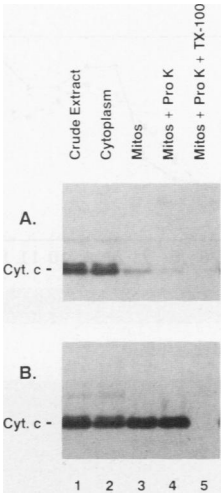


FIG. 2. Evidence that expression of high levels of heme lyase leads to efficient accumulation of apocytochrome *c* inside mitochondria. Subcellular fractionations and immunoblotting were performed as for Fig. 1, except that cells were cultured on minimal medium as described in Materials and Methods in order to maintain the multicopy plasmid containing the *CYC3* gene and electrophoresis was performed on a minigel. (A) Strain B-7911 (*cyc7-H3-67 CYC3*$^+$); (B) strain B-8118 (*cyc7-H3-67 CYC3*$^+$ [pAB790]). This strain is the same as B-7911 except that it has been transformed with the multicopy plasmid containing *CYC3* under control of the actin promoter. A total of 25 μg of protein was loaded on lanes 1 and 2, and 3 μg was loaded on lanes 3 to 5. Contents of the lanes are as described for Fig. 1.

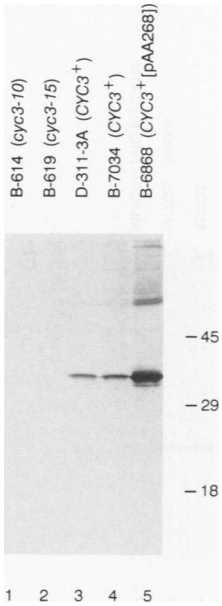


FIG. 3. Detection of heme lyase with antibodies produced by using a synthetic peptide corresponding to the predicted sequence of the carboxyl-terminal region. Extracts of the indicated strains were subjected to SDS-gel electrophoresis on 12% acrylamide gels (22) and immunoblotted as described in Materials and Methods. A total of 250 μg of protein was loaded in each lane. The *CYC3* alleles of each strain are indicated at the top. pAA268 is a multicopy plasmid containing the *CYC3* gene under control of the actin promoter (9). The mobilities of molecular weight markers (in kilodaltons) are indicated at the right.

10-fold increase in heme lyase levels in the plasmid-containing strain compared with the untransformed strain was evident on immunoblots of cell fractions from the two strains, both probed with anti-heme lyase antibodies (results not shown).

To test whether the observed apocytochrome *c* accumulation in mitochondria is reversible in vitro, the amounts of immunoreactive apocytochrome *c* inside mitochondria immediately following mitochondrial isolation (about 3 h after cell lysis; Fig. 1C and D, lanes 4 to 6) were compared with the amounts remaining internalized after 24 h at 0°C (lanes 7 to 9). Both entry of apocytochrome *c* into and egress of apocytochrome *c* from isolated mitochondria have been previously reported to be relatively unaffected by low temperature (15, 29). Since mitochondria were not reisolated during the 24-h incubation, exit of apocytochrome *c* across the outer membrane would be expected to cause a decrease in the amount of cytochrome *c* protected against protease, with the amount in the total mitochondrial fraction remaining constant. In fact, a decrease in the amount of cytochrome *c* in both the total and protease-protected fractions was observed, suggesting that degradation of the cytochrome *c* occurs during the incubation. No significant decrease in the protease-protected fraction compared with the total mitochondrial level could be detected in either the *cyc3* or *CYC3*$^+$ strains. This finding suggests that the low level of mitochondrial accumulation of altered apocytochrome *c* in strain B-7911 is not a residual level that could have been removed by more complete washing of mitochondria.

Strains B-7911, B-8076, and B-8077, with low levels of internal mitochondrial apocytochrome *c*, appeared to contain some precursor that was bound to mitochondria but not internalized. While the amount bound was variable, it was generally a few percent of total cellular apocytochrome *c*. Although we have not determined the specificity of such binding, it is unlikely to represent precursor bound to heme lyase in a transmembrane configuration as would be predicted from the results of Nicholson et al. (29), since it is seen in strains B-8076 and B-8077, which completely lack heme lyase (Fig. 1B and C, lane 4). If high-affinity binding sites for apocytochrome *c* exist on the mitochondrial surface, it is likely that they are saturated by the high levels of cytoplasmic apocytochrome *c* in strains that cannot attach heme to cytochrome *c*.

The total amounts of apocytochrome *c* in the spheroplast and crude extract fractions of the holo-cytochrome *c*-deficient strains B-8077, B-8076, and B-7911 (Fig. 1B to D, lanes 1 and 2) are lower than those in B-7908 (Fig. 1A). (Note that the amounts of protein loaded per lane in Fig. 1B to D are twice what was loaded in Fig. 1A.) Comparisons of immunoblots of purified apo-iso-2-cytochrome *c* and holo-iso-2-cytochrome *c* indicate that the antibodies recognized these two forms of the protein to approximately equal extents. Since neither *cyc3* mutations (11, 23) nor the alteration in the *CYC7* coding region would be expected to affect the transcriptional or translational efficiency of *CYC7*, the decrease in immunoreactive gene product most likely results from increased degradation of cytoplasmic apo-iso-2-cytochrome *c*, compared with mitochondrial holo-iso-2-cytochrome *c*. Strain B-8118, containing much of its apocytochrome *c* inside mitochondria, exhibits higher total apocytochrome *c* levels than did strains containing apocytochrome *c* localized in the cytoplasm (Fig. 2), suggesting that mitochondrial import, or binding to heme lyase, can

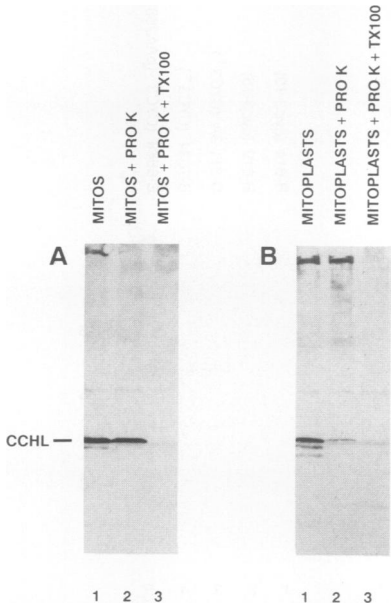


FIG. 4. Evidence that heme lyase is not accessible to protease external to mitochondria but is readily digested following disruption of the outer mitochondrial membrane. Mitochondria from the normal strain D311-3A were isolated and, where indicated, osmotically treated to disrupt the outer membrane as described in Materials and Methods. Intact or osmotically treated mitochondria at 0.8 mg/ml were centrifuged at 30,000 × *g* for 40 min at 4°C. The pellet fraction was resuspended to the original volume and treated with protease, where indicated, as described in Materials and Methods. SDS-gel electrophoresis and immunoblotting were as described in the legend to Fig. 1 except that 75 μg of protein was loaded in each lane. Contents of the lanes are essentially as described in the legend to Fig. 1. (A) Experiment performed with intact mitochondria; (B) experiment performed with mitoplasts. The position of cytochrome *c* heme lyase (CCHL) is indicated.

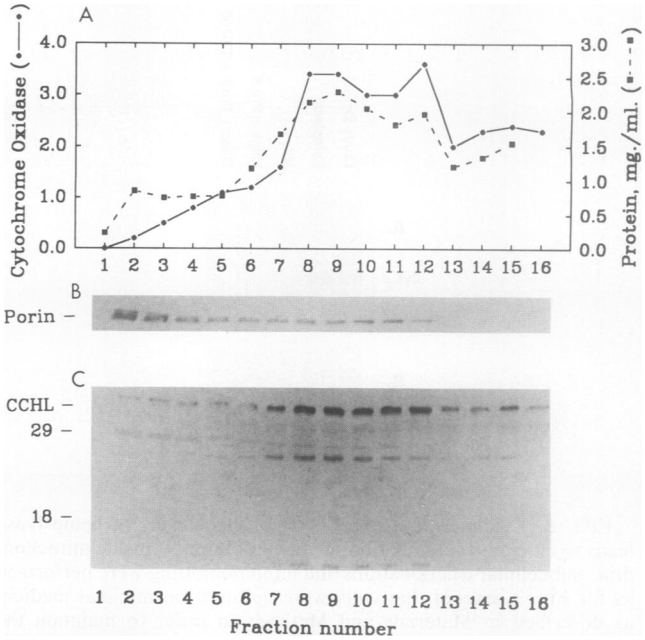


FIG. 5. Migration of heme lyase with inner mitochondrial membranes in sucrose gradient fractionations of disrupted mitochondria. (A) Mitochondria from the normal strain D311-3A were isolated, disrupted, and separated on 0.9 to 1.3 M sucrose gradients as described in Materials and Methods. Fractions were assayed for total protein (■) following a precipitation with trichloroacetic acid (34) and for cytochrome *c* oxidase (●). (B) A portion of each sample was subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting using antiporin antibodies. (C) A portion of each sample was subjected to electrophoresis and immunoblotting using anti-heme lyase antibodies. CCHL, migration of heme lyase in a mitochondrial extract from a yeast strain containing *CYC3*$^+$ expressed from a multicopy plasmid. The molecular weights (in thousands) of two additional markers are indicated at the left. The fractions from the top of the gradient are on the left.

stabilize apocytochrome *c* even when conversion to holocytochrome *c* is blocked. In previous studies, the half-life of cytoplasmic apo-iso-2-cytochrome *c* stability was determined to be at least 1 h. However, accurate turnover rates have not been determined for apo-2- or iso-2-cytochromes *c* (11).

**Submitochondrial localization of cytochrome *c* heme lyase.** Peptides corresponding to two regions of the predicted amino acid sequence of the *CYC3* gene product were synthesized and used to produce antibodies in rabbits. Antibodies against both of these peptides specifically recognized a band of the expected molecular weight of heme lyase on immunoblots of SDS-polyacrylamide gels of yeast extracts. The antibody raised against the carboxyl-terminal peptide appeared to bind more strongly and was used in the experiments presented here. The 30-kDa band recognized by this antibody was detectable on immunoblots of whole-cell extracts of normal strains (Fig. 3, lanes 3 and 4), was not detectable in extracts of two independently isolated *cyc3*$^-$ strains (lanes 1 and 2), and was enriched in extracts of a strain containing *CYC3* on a multicopy plasmid (lane 5). The higher-molecular-weight bands that are more pronounced in the extract of the strain containing the multicopy plasmid may be aggregates of heme lyase that are not disrupted by boiling in SDS.

When intact mitochondria were subjected to proteolysis with proteinase K, no digestion of heme lyase could be detected on immunoblots unless the mitochondrial membranes had previously been disrupted with detergent (Fig. 4A). This result demonstrated that at least the protease-sensitive domains of heme lyase are not exposed on the outer surface of mitochondria. If the outer mitochondrial membranes were selectively disrupted by resuspension in 0.1 M mannitol (6), extensive proteolysis of heme lyase was observed in the absence of detergent (Fig. 4B). Osmotic treatment of the mitochondria released 70% of the total mitochondrial activity of the soluble intermembrane space marker cytochrome $b_2$ to the supernatant. The latency of cytochrome $b_2$ activity, demonstrated by using membrane-impermeant horse holocytochrome *c* as a substrate and comparing the activity in the presence or absence of 0.1% Triton X-100 (6), was greater than 90% in unshocked mitochondria and was 30% in shocked mitochondria. In contrast, only 2% of the activity of a matrix marker, fumarase, was released by this treatment in 0.1 M mannitol. These results indicate that heme lyase is exposed to the intermembrane space. Furthermore, the enzyme remained associated with the mitoplasts following disruption of the outer membrane, suggesting that it is membrane associated. Similar results were obtained on immunoblots probed with antibody raised against the internal peptide of heme lyase (not shown). This

finding demonstrates that the proteolysis observed on immunoblots is not simply the removal of the extreme carboxyl-terminal amino acid residues.

To identify the mitochondrial membranes with which heme lyase is associated, a mitochondrial subfractionation was performed, using osmotic swelling followed by reshrinking and sonication. Following removal of unbroken mitochondria, membranes were subjected to ultracentrifugation on a sucrose gradient under conditions that have been reported to allow separation of inner membrane, outer membrane, and sites of contact between the two membranes (35). Immunoblotting with antiporin antibodies was used to identify outer membrane-containing fractions. Cytochrome *c* oxidase activity was monitored as a marker of inner membranes. Three turbid bands could be seen on the gradients, and three peaks of total protein could be detected in the fractions (Fig. 5). From the distribution of markers, the least dense of these peaks represents outer membranes, the peak of intermediate density contains contact sites, and the densest peak contains inner membranes, as reported previously (35). Immunoblotting of fractions with anti-heme lyase antibodies revealed that this enzyme is present in the two densest peaks but not in outer membranes. The distribution of heme lyase appears to match closely that of cytochrome oxidase, suggesting that it is not specifically enriched at contact sites compared with overall outer membrane. Some proteolysis of heme lyase was observed during this submitochondrial fractionation, leading to the appearance of lower-molecular-weight immunoreactive bands that are not seen in immunoblots of intact mitochondria. However, intact heme lyase remains the predominant species, and the distribution of fragments in the sucrose gradient fractions is similar to that for the intact protein. The faint band at 29 kDa in the anti-heme lyase immunoblot appears to correspond to porin, which is a very abundant Coomassie blue-staining band in the outer membrane-containing fractions.

## DISCUSSION

The existence of a mechanistic coupling between the covalent attachment of heme to apocytochrome *c* and import of the protein into mitochondria has been suggested by a number of in vitro experiments. Hennig and Neupert (19) showed that the heme analog deuterohemin, when present in excess, could block import of newly synthesized apocytochrome *c* into isolated *Neurospora* mitochondria. Isolated yeast or *Neurospora* mitochondria that lack heme lyase were unable to import apocytochrome *c* (10, 28). However, a recent report, also based on in vitro studies, has suggested that at least the initial stages of import are unrelated to heme attachment (15).

Mutant yeast and *Neurospora* strains have been isolated that lack holocytochrome *c* in vivo because of a deficiency in heme lyase (9, 28) or because they contain altered forms of cytochrome *c* with amino acid substitutions at the site of covalent heme attachment (17). However, the instability of the apo form of iso-1-cytochrome *c*, the predominant isozyme in *S. cerevisiae* (11), or of the only apocytochrome *c* in *N. crassa* (28) made it difficult to determine whether the defect in heme attachment also resulted in a defect in import. Fusion proteins, in which the cytochrome *c* was coupled to reporter genes, were found to be capable of efficient targeting to the mitochondrial surface in the absence of heme attachment, but membrane translocation of the fusion proteins was impaired when heme could not be attached (32). We have circumvented the problem of the instability of apocytochrome *c* and the need to use fusion proteins by studying the subcellular distribution of cytochrome *c* in yeast strains that contain a deletion of the gene encoding iso-1-cytochrome *c* and express high levels of iso-2-cytochrome *c*, for which the apoprotein has been shown to be stable (11).

In yeast strains containing normal levels of heme lyase and the normal amino acid sequence for iso-2-cytochrome *c*, 95% of cellular cytochrome *c* detected by immunoblotting is found in the mitochondrial fraction. The small amount in other fractions is no greater than the level of cross-contamination measured using cytochrome $b_2$, another enzyme of the intermembrane space. On the other hand, in strains that lack heme lyase or contain normal levels of heme lyase but express an altered iso-2-cytochrome *c* with amino acid substitutions that block heme attachment, about 95% of cytochrome *c* is in the cytoplasm. Although the total amount of immunologically detectable cytochrome *c* in these holocytochrome *c*-deficient strains is less than in control strains, the substantial increase in cytochrome *c* measured in the cytoplasmic fraction ensures that the results cannot be explained by degradation of mitochondrial apocytochrome *c*. These results clearly establish a role for interaction between heme lyase and apocytochrome *c* in the accumulation of cytochrome *c* in mitochondria in vivo.

In strain B-7911 (*cyc7-H3-67 CYC3*$^+$) containing normal levels of heme lyase, a low but significant level of import was observed for altered iso-2-cytochrome *c* containing serine residues substituted for the cysteine residues at the heme attachment site. In vitro, we had previously detected inefficient import of altered iso-1-cytochrome *c* containing single amino acid substitutions at the cysteines involved in heme binding or at the adjacent histidine, but it had not been verified that these substitutions completely block heme attachment. On the other hand, apo-iso-2-cytochrome *c* that was modified at the cysteines with *N*-ethylmaleimide could not be imported into isolated mitochondria to any detectable extent (10).

The *cyc3*-Δ strains B-8076 and B-8077, lacking heme lyase, reproducibly contained even lower levels of mitochondrial apocytochrome *c* than did the *CYC3*$^+$ strain B-7911, which expressed mutant iso-2-cytochrome *c* that could not have heme attached. Because of the extent of cytoplasmic contamination of the isolated mitochondria, it was not possible to determine whether these *cyc3*-Δ strains contained any mitochondrial apocytochrome *c* at all. Mitochondria isolated from heme lyase-deficient cells had previously been found to be unable to import detectable cytochrome *c* in vitro (10). On the other hand, overexpression of heme lyase in the strain B-8118 led to accumulation of high levels of altered *cyc7-H3-67* apocytochrome *c* inside mitochondria. Thus, the amount of heme lyase present can strongly affect the efficiency of cytochrome *c* accumulation in mitochondria, even when attachment of heme to the protein catalyzed by heme lyase is blocked.

Recently, Margoliash and coworkers presented evidence that apocytochrome *c* can be reversibly imported into isolated mitochondria, suggesting that the early steps of translocation do not depend on heme attachment (15, 41). These authors report that apocytochrome *c* can be efficiently concentrated inside mitochondria, even when the protein is altered by the substitution of serine residues for the cysteine residues that are involved in the thioether linkages to heme. In contrast, we find that in yeast cells with the normal complement of heme lyase, only a small proportion of apocytochrome *c* is localized inside mitochondria if heme is

not attached and that the concentration of apocytochrome *c* inside mitochondria does not exceed that in the cytoplasm. Detailed comparison of the results presented here with those of Hakvoort et al. (15) is difficult because of the differences between the experimental systems. Hakvoort et al. studied import of in vitro-synthesized *Drosophila melanogaster* and rat cytochrome *c* into isolated mouse liver mitochondria, mediated by a cytoplasmic factor from wheat germ, a system which, under optimum conditions, converts a maximum of 5% of the added precursor to holocytochrome *c*. In our studies, by immunoblotting subcellular fractions, we are attempting to determine steady-state distributions in living yeast cells that normally convert virtually all of their cytochrome *c* to the holo form (25). One risk in our approach is that cytochrome *c* might leak out of mitochondria during subcellular fractionation. The appearance of apocytochrome *c* in the cytoplasmic fraction from strains that are defective in heme attachment rules out the possibility that the apparent depletion of mitochondrial cytochrome *c* in these strains results from apocytochrome *c* leakage from mitochondria during the repeated centrifugation steps required for mitochondrial isolation. We cannot, however, completely eliminate the possibility that apocytochrome *c* is escaping into the cytoplasm at 4°C during the 15 to 30 min that elapse between the time we homogenize the spheroplasts and the first centrifugation that separates mitochondria from cytoplasm. For such release of cytochrome *c* to be occurring, the rate of leakage would have to be faster than that seen by Hakvoort et al. (15). In addition, the estimated concentrations of cytoplasm and precursor following cell disruption in our procedure are even higher than those used in the in vitro import assays of Hakvoort et al. (15).

Previously, the subcellular location of heme lyase was investigated by fractionation of the activity of this enzyme. However, Nicholson et al. (30) found that total activity was lost as mitochondria were disrupted or cofactors were lost during fractionation. Thus, we have made use of specific antibodies recognizing heme lyase to monitor the polypeptide chain during mitochondrial fractionation. We find that the enzyme is not exposed to any significant extent on the external mitochondrial surface but instead is readily accessible to the intermembrane space. Furthermore, heme lyase fractionates with inner mitochondrial membranes following disruption of mitochondria and does not appear to be enriched in contact sites between the inner and outer membranes compared with the inner membrane marker, cytochrome *c* oxidase. The association with the inner membrane is in agreement with the findings of Enosawa and Ohashi (13) for *S. cerevisiae* but partially contradicts those of Nicholson et al. (30), in which loss of heme lyase activity appeared to correlate with loss of soluble markers of the intermembrane space of *Neurospora* mitochondria. More recent studies by Nicholson et al. (29) found heme lyase to be membrane associated but did not differentiate between the inner and outer membrane. It is unlikely that heme lyase is an integral protein of the inner mitochondrial membrane, given the hydrophilic nature of the predicted amino acid sequence of the protein (9).

The results presented here demonstrate that cytochrome *c* heme lyase, an enzyme that plays a role in accumulation of cytochrome *c* in the space between the two mitochondrial membranes, is associated with the outer surface of the inner mitochondrial membrane. How can an enzyme on the inner membrane mediate transport across the outer membrane? One possibility is that the enzyme could be localized to sites of contact between the inner and outer membranes. Even

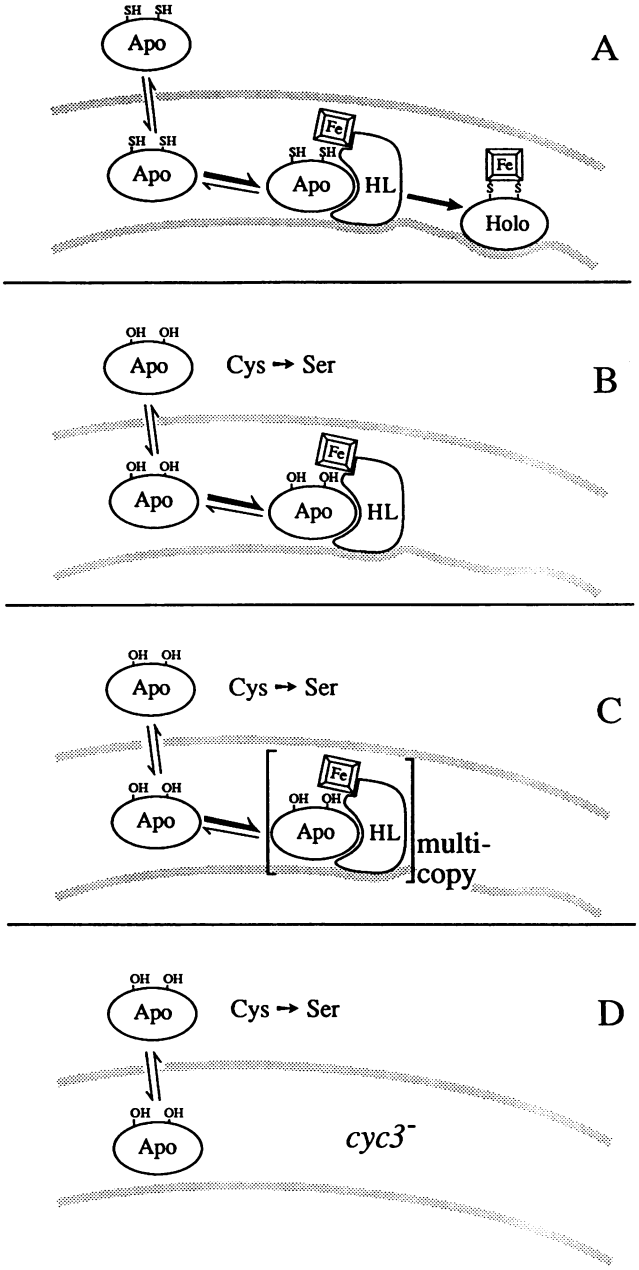


FIG. 6. Model for cytochrome *c* import into mitochondria. In this hypothetical scheme, apocytochrome *c* (Apo) diffuses reversibly across the outer mitochondrial membrane, either through the lipid phase or through a proteinaceous pore, and then binds to cytochrome *c* heme lyase (HL). (A) Attachment of heme would trigger a conformational change trapping holocytochrome *c* (Holo) in the intermembrane space and promoting binding to the inner membrane. (B) In the absence of an appropriate heme attachment site on apocytochrome *c*, this binding could still provide some intramitochondrial accumulation. (C) Expression of high levels of heme lyase leads to increased accumulation of apocytochrome *c*. (D) No import is detected if heme lyase is completely absent.

though heme lyase does not appear to be preferentially localized at such sites, we cannot rule out the possibility that the fraction of the population that is present at contact sites is the fraction that is important in mediating import. In this

case, varying the amount of heme lyase would affect import by changing the abundance of the transport machinery. However, a more likely explanation is that heme lyase is not involved in the actual transport across the outer membrane but instead serves to trap imported apocytochrome *c* in the intermembrane space as shown in Fig. 6. Since cytochrome *c* undergoes a substantial conformational change upon having heme attached (14, 42), heme lyase could serve to convert normal cytochrome *c* from a transport-competent form to a trapped form. In the case of the altered apocytochrome *c* that cannot have heme attached, heme lyase could promote mitochondrial accumulation by directly binding apocytochrome *c*. Since heme lyase is not normally an abundant molecule (9), this would allow only low levels of import, as is observed. However, in strain B-8118 expressing high levels of heme lyase, as shown in Fig. 2, this leads to efficient accumulation of the altered apocytochrome *c*.

An implicit assumption of this model is that cytochrome *c* is transported across the outer mitochondrial membrane by a reversible mechanism, perhaps the spontaneous insertion of precursor into the membrane as seen in biophysical studies (12, 37). The simplest interpretation of our results is that such reversible transport is a diffusionlike process, since we can detect no import in the absence of trapping by heme lyase and since apocytochrome *c* bound to heme lyase could not be washed out of mitochondria. In the model presented in Fig. 6, heme lyase plays no direct role in binding of apocytochrome *c* to mitochondria or in translocation of the precursor across the outer mitochondrial membrane. This appears to conflict with the recent suggestion from in vitro import experiments that heme lyase serves as a receptor for external binding of apocytochrome *c* to mitochondria (29). These authors find that apocytochrome *c* forms a complex with heme lyase while the precursor is still accessible to protease on the external surface of mitochondria and that such external binding is greatly reduced in a heme lyase-deficient compared with a normal strain. In our studies, we do detect some apocytochrome *c* bound to the outside of mitochondria under conditions in which heme attachment is blocked; however, this binding is low compared with the overall cellular apocytochrome *c* levels, and it appears to be approximately the same in *CYC3*$^+$ and *cyc3*-Δ strains.

The results presented here confirm, in an in vivo system, that the covalent attachment of heme can promote accumulation of cytochrome *c* in mitochondria. They also demonstrate, for the first time in an in vivo system, that apocytochrome *c* can be imported into mitochondria in the absence of covalent heme attachment. Although such import does not involve the catalytic activity of cytochrome *c* heme lyase, we find that it is nonetheless dependent on the presence of the enzyme, since it does not occur in strains lacking heme lyase and occurs very efficiently in strains that express high levels of heme lyase. Together with the evidence we have provided that heme lyase is associated with the inner mitochondrial membrane, these findings are consistent with a model whereby apocytochrome *c* can be reversibly transported across the outer mitochondrial membrane and then is trapped, either by being converted to holocytochrome *c* or by binding to heme lyase. The mechanisms of transport across the outer membrane, however, remain to be identified. The demonstration of efficient import in vivo in the absence of heme attachment should allow the membrane transport step to be studied with less interference from the heme attachment step than could previously be achieved.

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