In Vivo Expression and Mitochondrial Targeting of Yeast Apoiso-1-Cytochrome *c* Fusion Proteins

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To define the import pathway for apoiso-1-cytochrome *c* in vivo, the coding region for bacterial chloramphenicol acetyltransferase (CAT) or yeast copper metallothionein (CuMT) was fused to the carboxy terminus of the apoiso-1-cytochrome *c* (iso-1) coding region. When the resulting iso-1/CAT and iso-1/CuMT fusion proteins were individually expressed in *Saccharomyces cerevisiae*, they were specifically targeted to the mitochondria and protected from trypsin digestion. Although iso-1/CAT was accessible to heme modification, it remained membrane associated because of the folded conformation of the CAT domain. A small deletion disrupting CAT structure resulted in the translocation of the resulting fusion protein, iso-1/CAT Δ , to the intermembrane space, where it functioned efficiently in respiratory electron transfer. Similarly, iso-1/CuMT was heme modified and nearly identical to iso-1 in its ability to support respiratory growth, indicating that the CuMT domain was compatible with translocation to the IMS. Inclusion of copper in the growth medium, which converts the loosely structured apo-CuMT to a tightly folded holo-CuMT, inhibited both heme attachment and respiratory growth without affecting mitochondrial targeting. Thus, by altering the folded conformation of the reporter moiety of these fusion proteins, it was possible to differentiate between those molecules arrested at the mitochondrial targeting step of the cytochrome *c* import pathway and those translocated to the intermembrane space. By replacing the heme-binding cysteine residues with serines, this system was used to demonstrate that the import requirement for heme attachment operated at the level of membrane translocation and not on mitochondrial targeting in vivo.

Cytochrome *c* is one of many nuclear gene products that are synthesized in the cytoplasm and function as part of the mitochondrial respiratory apparatus (reviewed in reference 6). Its import pathway has been defined mainly through the use of in vitro systems (see reference 34 for references). The apocytochrome *c* is covalently modified with heme, and the holocytochrome *c* is localized to the mitochondrial intermembrane space (IMS). Cytochrome *c* differs from most other nucleus-encoded respiratory proteins in that it is synthesized without a cleavable amino-terminal signal presequence (28, 32). In contrast to holocytochrome *c*, which is a highly structured spherical molecule (5), the apoprotein lacks defined secondary structure (36). Folding of the apoprotein around the heme prosthetic group during import presumably drives the translocation reaction forward and also makes it irreversible (13). The cytochrome *c* heme lyase is membrane bound but inaccessible to externally added proteases (24) and may be involved in the specific binding of the apoprotein to the mitochondria (24, 34). An important question that has not been systematically addressed is the identity of the apoprotein determinants required for membrane recognition and insertion in vivo.

Fusion proteins with various reporter molecules have been useful in elucidating the import determinants for nucleus-encoded mitochondrial proteins whose import is directed by an amino-terminal presequence (6). One important property of reporter molecules used in the construction of fusion proteins is whether they have a structure compatible with passage through a biological membrane. Proteins having an unfolded conformation are import competent, while those

having a tightly folded three-dimensional structure are often blocked in membrane translocation (3, 9).

To develop an in vivo system for mapping import determinants on cytochrome *c*, either the bacterial chloramphenicol acetyltransferase (CAT) or the *Saccharomyces cerevisiae* copper metallothionein (CuMT) reporter protein was fused to the carboxy terminus of apoiso-1-cytochrome *c*. The resulting iso-1-cytochrome *c*-CAT (iso-1/CAT) and iso-1-cytochrome *c*-CuMT (iso-1/CuMT) fusion proteins were expressed in *S. cerevisiae*. CAT is a stable globular protein (30), while the folding of apo-CuMT to a stable holoprotein is known to require the binding of copper ligands (38). Fusion proteins with either reporter molecule were correctly targeted to mitochondria in vivo, but only those with an unfolded conformation were able to efficiently traverse the mitochondrial outer membrane and support respiratory growth. The results establish that information in apoiso-1-cytochrome *c*, when expressed as part of a fusion protein, can direct mitochondrial import and that fusion protein structure is compatible with the biological function of cytochrome *c*. In the accompanying paper (25), we take advantage of the ability of fusion proteins to target to mitochondria in vivo to map the structural domains on apoiso-1-cytochrome *c* required for mitochondrial membrane recognition and insertion.

MATERIALS AND METHODS

Strains. *S. cerevisiae* NA1 (*MATa leu2-3 leu2-112 his4-34 ura3-52 URA3 HIS4-lacZ*) was derived from strain F45 (*MATa leu2-3 leu2-112 his4-34 ura3-52*) (29) by integrating a *HIS4-lacZ* fusion gene (20) at the *URA3* locus. Strain LSR1 (*MATa leu2-3 leu2-112 his4-34 ura3-52 hem1*), deficient in δ -aminolevulinic acid (δ -ALA) synthase, was obtained from the haploid progeny of a mating between GT39-6B (*MATa*

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adel hem1) (39) and F45. Strain N1B (*MATa leu2-3 leu2-112 his4-34 ura3-52 cyc1-1*) is deficient in iso-1-cytochrome *c* because of a deletion in *CYC1* (29). Strains were transformed with expression vectors as described elsewhere (15) with the *LEU2* selectable marker. Cell growth was monitored with a Klett-Summerson colorimeter as described previously (29).

DNA methods and construction of plasmids. Fusion proteins were expressed by using a derivative of the yeast shuttle vector pAAH5 (1). To construct YEp*CYC1/cat* for expression of iso-1/CAT, the carboxy terminus of the *CYC1* coding region was fused to the *cat* coding region through a *Clal* site following M13 site-directed mutagenesis (18) of the *CYC1* termination codon with the oligonucleotide 5'-GGC CTGTTGACTCACAG-3'. For cloning of *CYC1-cat* into pAAH5, the *Bam*HI-*Hind*III *ADC1* promoter fragment was modified with a synthetic *Hind*III-*Eco*RI adaptor which included the translational initiator AUG codon and the first four bases of *CYC1*, while the *Hind*III site of the *ADC1* terminator from pAAH5 was converted to *Bgl*II with a synthetic linker. YEp*CYC1/catΔ*, for expression of iso-1/CATΔ, was constructed by deleting codons for amino acids 72 to 106 in the CAT domain between in-frame *Mae*III restriction sites. Conversion of cysteine codons 19 and 22 to serine codons in the *CYC1* coding region of YEp*CYC1/cat* was accomplished by M13 site-directed mutagenesis (18) with oligonucleotide 5'-CGGTGTGGGATTGTAGAGATCT AGTC-3'. Serine codons at these positions were introduced into YEp*CYC1/catΔ* by substituting the *Eco*RI-*Clal* coding region fragment containing these mutations.

YEp*cat* was constructed by ligating the 5' end of the *cat* coding region (*Hind*III-*Eco*RI) from pSV0CAT (10) to a *cat-ADC1* terminator fragment (*Eco*RI-*Sph*I) and cloning into the pAAH5 *Bam*HI-*Sph*I vector containing the *ADC1 Bam*HI-*Hind*III promoter fragment. YEp*bcat*, for expression of bridge-CAT protein (bCAT), was constructed by modifying the *Clal* site of the *Clal-Bgl*II *cat* fragment to *Eco*RI and then ligating it downstream of the *ADC1* promoter-ATG linker fragment in the pAAH5 *Bam*HI-*Sph*I vector.

The CAT coding region (*cat*) was replaced with the CuMT gene (*CUP1*) (2) in the expression cassette to generate YEp*CYC1/CUP1* for the expression of iso-1/CuMT. The *Rsa*I fragment from *CUP1* minus the promoter sequences was simultaneously modified with *Clal* and *Sph*I linkers. The resultant *Clal-Sph*I fragment was cloned into the expression vector along with the complete *CYC1* coding region under the regulation of the *Bam*HI-*Eco*RI *CYC1* promoter sequences (32).

For expression of iso-1-cytochrome *c*, YEp*CYC1* was constructed by converting the *Bam*HI-*Hind*III fragment containing the entire *CYC1* gene (32) to *Bam*HI and cloning into the *Bam*HI site of a derivative of pAAH5 deleted of *ADC1* promoter and terminator fragments. All site-directed modifications were verified by M13 dideoxy sequence analysis (27).

Immunological methods. Polyclonal antibody was raised in a New Zealand White female rabbit immunized with 200 μg of CAT (Sigma Chemical Co.) per injection during a series of subcutaneous and intramuscular injections. Polyclonal antibodies to total yeast cytochrome *c* were raised as described previously (29). Cross-reactive antibodies in both antisera were absorbed out by using total protein extracts from strains NA1 (CAT antibodies) and N1B (yeast cytochrome *c* antibodies) bound to a Nytran-modified nylon 66 membrane (Schleicher & Schuell). The anti-CAT antiserum was further purified by affinity chromatography (Affinity Chromatography Principles and Methods; Pharmacia). Polyclonal anti-

body to β-galactosidase was obtained from Sigma, while the F₁-ATPase β-subunit antibody was provided by Michael G. Douglas.

Cytochrome *c*, CAT, β-galactosidase, and iso-1/CAT were immunologically detected in total protein extracts as described previously (29) except that the sodium dodecyl sulfate (SDS)-dissociated and boiled samples were equilibrated in 25 mM Tris hydrochloride (Tris HCl, pH 7.5)–0.2% SDS–1 mM phenylmethylsulfonyl fluoride (PMSF)–5 mM EDTA with Centricon-10 concentrators (Amicon). Samples were electrophoresed, and the proteins were transferred to Nytran with a Hoefer TE51 Transphor device (Hoefer Scientific Instruments). The membrane was treated with a mixture of antibodies, and the proteins were visualized with 4-chloro-1-naphthal (Sigma).

Subcellular fractionation. Mitochondria were isolated from cultures grown to late log phase (approximately 100 Klett units) in 1% DL-lactate containing medium (31) yielding about 2 g (wet weight) of cells per liter. Glusulase was used to convert the harvested cells to spheroplasts (7). Following a gentle wash in 1 M sorbitol, the spheroplasts were Dounce homogenized in buffer A (20 mM tricine-KOH [pH 7.4], 0.6 M sorbitol, 10 mM KCl, 1 mM dithiothreitol) containing 1 mM PMSF and fractionated via differential centrifugation by previously described methods (4, 7, 26). A portion of the cytoplasmic supernatant was concentrated and washed in a chilled Centricon-10 concentrator at 5,000 × *g* for 1 h at 4°C with 20 mM tricine-KOH (pH 7.4)–10 mM KCl–1 mM dithiothreitol–1 mM PMSF and used to determine CAT activity. The 17,000 × *g* mitochondrial pellet was gently washed and suspended in a small volume of buffer A. For isolation of a microsomal fraction, the postmitochondrial supernatant was centrifuged at 17,000 × *g* for 15 min to remove residual mitochondria. Microsomes were pelleted by centrifugation at 100,000 × *g* for 90 min at 2°C as described previously (26), suspended in a small volume of buffer A, and used immediately in protease protection experiments.

For determination of fusion protein uptake, 150 μg of freshly isolated crude mitochondria or microsomes was diluted to 2.0 mg/ml with buffer A and subjected to trypsin (Sigma) digestion (100 μg/ml) at 30°C for the desired period of time (12). The reaction was terminated with the addition of 1 mM PMSF and loading dye, followed by heating at 90°C for 3 to 5 min. The products were electrophoresed on a 15% polyacrylamide-SDS gel and identified by immunoblotting as described above.

Submitochondrial fractionation. Mitochondrial fractions were diluted to 2.0 mg/ml with buffer A and then treated with trypsin (10 μg/ml) for 5 min at 30°C to remove cytoplasmic contamination. The reaction was terminated with the addition of soybean trypsin inhibitor (50 μg/ml), and the trypsinized mitochondria were washed with buffer A containing 1 mM PMSF plus soybean trypsin inhibitor (included in all subsequent fractionation steps). To prepare mitoplasts, these mitochondria (5.0 mg/ml) were diluted 10-fold in buffer A minus sorbitol (12) and gently stirred on ice. Following centrifugation at 17,000 × *g* for 15 min at 4°C, the first IMS fraction was removed with the supernatant from the mitoplast pellet. Yeast cytochrome *c* was effectively removed from the mitoplast fraction by salt washing in 0.15 M KCl solution (16). Following centrifugation at 17,000 × *g* for 15 min at 4°C, the second IMS supernatant fraction was combined with the first and washed with 10 mM Tris-HCl (pH 7.4) in a Centriprep-10 (Amicon) concentrator. The mitochondrial matrix was separated from the total membranes in the mitoplast fraction by established procedures (4) except

that the mitoplast fraction was disrupted with a Branson sonifier at setting 2.5 for 130 s at 30% duty output. The matrix fraction was concentrated like the IMS fraction above, while the membrane fraction was suspended in 10 mM Tris-HCl (pH 7.4)–1 mM PMSF. Each submitochondrial fraction was analyzed by immunoblotting as described above.

Cell labeling and immunoprecipitation. For detecting heme-labeled fusion proteins in strain LSR1, precultures were grown in DL-lactate-containing medium supplemented with 40 μ M δ -ALA (39) (Sigma) and used to inoculate 5 ml of DL-lactate medium supplemented with 40 μ M 50-mCi/mmol 5-amino[4- 14 C]levulinic acid hydrochloride (Amersham). For detecting heme-labeled iso-1/CuMT in N1B, cultures were grown as above except without δ -ALA in the preculture medium. All cultures were grown to late log phase (approximately 100 Klett units), and cells were harvested and ground on ice in cold breaking buffer (20 mM NaPO₄ [pH 7.2], 10 mM MgCl₂, 10 mM β -mercaptoethanol, 200 mM NaCl) containing 1 mM PMSF. SDS and Triton X-100 were added as previously described (35) to solubilize the membranes. Following incubation in a 100°C water bath for 5 min, the extract was clarified by centrifugation in a microfuge for 30 min, and the protein concentration was determined (19). Each extract was immunoprecipitated with anti-yeast cytochrome *c* (150 μ l of a 1:1 dilution) and anti-CAT (50 μ l of a 1:1 dilution) antisera preabsorbed as described above. Antiserum was added to the protein extract and incubated for at least 12 h at 4°C with gentle mixing. The antigen-antibody complexes were collected by addition of 150 μ l of protein A-agarose (Boehringer Mannheim Biochemicals), followed by gentle mixing at 4°C for a minimum of 4 h. Precipitates were collected by centrifugation in a microfuge for 10 s and washed four times with 500 μ l of 20 mM NaPO₄–0.5 M NaCl–0.1% SDS–1% Triton X-100. The washed immune complexes were then dissociated from the protein A-agarose by boiling in loading dye for 3 min and centrifuged for 10 s in a microfuge, and the supernatant was electrophoresed on a 15% SDS–polyacrylamide gel. The gel was stained with Coomassie brilliant blue and destained, and the labeled proteins were detected by fluorography.

Protein and enzyme assays. The protein concentration of total extracts, mitochondrial fractions, and submitochondrial fractions was determined by the method of Lowry et al. (19). CAT (10) and β -galactosidase (22) activities were determined by previously established methods. Densitometric scanning of autoradiographs was performed with a Zeinah soft laser densitometer combined with a Hewlett-Packard 3390 integrator.

RESULTS

Expression of an iso-1/CAT fusion protein in yeast cells. Figure 1 shows the yeast shuttle vector and the various cassettes used for the expression of iso-1/CAT (YEpcYC1/cat) and control proteins CAT (YEpcat) and bCAT (YEpbcat). The latter contains the 18-amino-acid bridge sequence used to facilitate the separation of the iso-1 and CAT protein domains. These vectors were derived from pAAH5 (1) and contain a *LEU2* selectable marker and 2 μ m plasmid origin of replication. Each hybrid gene is under the transcriptional control of the yeast *ADC1* promoter. With a similar construct, rat cytochrome *c* was previously found to be expressed in yeast cells at a level approximately equal to the normal derepressed level of iso-1-cytochrome *c* (29). Transformants containing these vectors were resistant to

FIG. 1. Summary of expression vectors. Cassettes for expression of iso-1/CAT (YEpcYC1/cat), bCAT (YEpbcat), CAT (YEpcat), cytochrome *c* (YEpcYC1), and iso-1/CuMT (YEpcYC1/CUP1) were cloned into the yeast shuttle vector derived from pAAH5 (narrow line) as indicated. *ADC1* promoter and terminator (stippled box) and iso-1-cytochrome *c* coding (open box) and CAT coding (solid box) sequences are indicated for each cassette. Forward-hatched boxes depict the 18-amino-acid (SIEGSGSLARFSGA KDAK) and 13-amino-acid (IDGQSNQSSHK) bridge sequences separating CAT, bCAT, or CuMT domains, respectively, from iso-1-cytochrome *c*. Back-hatched boxes represent the noncoding and flanking regions of the *CYC1* gene, and cross-hatched boxes depict the *CUP1* sequences. Restriction enzymes: B, *Bam*HI; H, *Hind*III; R, *Eco*RI; C, *Cla*I; b, *Bgl*II; S, *Sph*I; P, *Pst*I.

chloramphenicol (1,500 μ g/ml), confirming that the CAT domain was expressed in its catalytically active form and that the hybrid genes were in frame. The wild-type strain and the transformant containing an out-of-frame hybrid gene expressed from the vector YEpcYC1/cat(OF) (Fig. 2) were sensitive to the antibiotic (not shown).

Apoiso-1-cytochrome *c* is unstable and does not accumulate in the cytoplasm (21). To determine whether a stable fusion protein of the predicted size of 39.6 kDa was being expressed, immunoblot analysis of SDS-dissociated total-protein extracts was performed (Fig. 2). To serve as an easily assayable cytosolic control protein, bacterial *lacZ* was integrated at the *URA3* locus under the control of *HIS4*, resulting in the expression of β -galactosidase in the host strain NA1. The expression of iso-1/CAT was therefore compared with that of cytoplasmic β -galactosidase and mitochondrial cytochrome *c* as internal controls. The immunoblot identified iso-1/CAT (39.6 kDa), CAT (25.3 kDa), and bCAT (27.3 kDa) at the expected sizes while no fusion protein was detected either in the host strain alone (no vector) or in a transformant where the iso-1-cytochrome *c* and CAT coding regions were out of frame [YEpcYC1/cat(OF)].

Iso-1/CAT specifically targeted to mitochondria and protected from external protease. To determine whether iso-1/CAT was associated with the mitochondria, yeast cell extracts were separated into crude mitochondrial and postmitochondrial fractions, and their CAT activities were compared with β -galactosidase as a cytoplasmic control (Table 1). Cytosolic contamination of the mitochondrial

FIG. 2. Expression of iso-1/CAT in yeast cells. SDS-dissociated and boiled extracts (100 μ g per lane) from NA1 transformant strains were electrophoresed on 15% SDS-polyacrylamide gels and immunoblotted with a mixture of antibodies raised to yeast cytochrome *c*, CAT, and β -galactosidase as described in Materials and Methods. The expression vector in each transformant (depicted in Fig. 1) is designated above each lane, and the positions of fusion and control proteins expressed in each transformant are indicated on the left (β -gal, β -galactosidase; cyt *c*, cytochrome *c*). YEPCYC1/cat(OF) refers to a derivative of YEPCYC1/cat in which the CAT and cytochrome *c* coding regions are out of frame. Numbers at the right indicate the molecular masses of protein standards (in kilodaltons).

fractions as measured by β -galactosidase activity was negligible (2 to 3%) and similar to the level of CAT activity (7%) observed in the bCAT control. In contrast, there was a fourfold enrichment of CAT activity in the mitochondrial fractions of strains expressing iso-1/CAT over those expressing bCAT. By this criterion, a significant fraction of iso-1/CAT was associated with the mitochondria.

To distinguish between mitochondrial uptake and nonspecific binding to other cellular membranes, mitochondrial and microsomal fractions were isolated and treated over a time course with trypsin, and the proteins were visualized by immunoblotting (Fig. 3). Iso-1/CAT was readily detected in the mitochondrial fraction, and the majority remained stable to trypsin treatment compared with the untreated control for at least 60 min (Fig. 3A). Protease resistance depended upon an intact membrane structure, because iso-1/CAT was trypsin sensitive in the presence of the nonionic detergent Triton X-100 (Fig. 3A, +T). Although fusion protein was also detected in a microsomal fraction, none of this material was resistant to trypsin (Fig. 3B), indicating that stable targeting of the fusion protein was specific to mitochondria and not to other cellular membranes.

The cytochrome *c* present in microsomal fractions is usually attributed to its redistribution during cell lysis (17).

TABLE 1. Subcellular localization of iso-1/CAT^a

Vector	β -Galactosidase activity ^b (%)		CAT activity ^b (%)	
	Mito	PS	Mito	PS
YEPCYC1/cat	3	97	26	74
YEpbcat	2	98	7	93

^a The distribution of β -galactosidase and CAT activity in mitochondrial (Mito) and postmitochondrial supernatant (PS) fractions was determined from NA1 transformant cultures grown at 30°C to late log phase in medium containing DL-lactate. The fractions were isolated by differential centrifugation as described in Materials and Methods.

^b 100% activity is defined as the sum of the recovered β -galactosidase or CAT activity units in both fractions.

FIG. 3. Specific protease protection of iso-1/CAT in isolated mitochondria. Crude mitochondrial (A) and microsomal (B) fractions isolated from transformants of NA1 expressing iso-1/CAT were either untreated (U) or treated with trypsin (100 μ g/ml) for the indicated times (in minutes) at 30°C. +T indicates pretreatment with 0.1% Triton X-100 prior to protease digestion. Following proteolysis, the fractions were electrophoresed on 15% SDS-polyacrylamide gels and immunoblotted with a mixture of antisera to yeast cytochrome *c* (cyt *c*) and CAT. (C) Mitochondrial fractions from strain NA1 expressing bCAT and treated with trypsin as in panel A. (D) Purified preparations of CAT (0.15 μ g) and yeast cytochrome *c* (0.5 μ g) were combined and treated with trypsin for the indicated times and subjected to immunoblotting.

The bCAT protein associated with untreated mitochondria was also trypsin sensitive within 15 min of treatment (Fig. 3C) and thus nonspecifically bound to the outside. The trypsin-sensitive molecules were degraded to a CAT-sized protein that remained trypsin resistant even in the presence of detergent. Similarly, the endogenous holocytochrome *c* was trypsin resistant under these conditions. The inherent stability of these molecules to trypsin was confirmed by treatment of purified CAT and holocytochrome *c* (Fig. 3D) and is probably the result of their compact globular structures.

Iso-1/CAT blocked in translocation to the IMS. In vitro studies suggested that covalent heme modification is coupled to the import of cytochrome *c* (8, 23, 24). To determine whether iso-1/CAT was covalently heme modified, the fusion protein was expressed in a *hem1* strain deficient in δ -ALA synthase and thus unable to synthesize heme in the absence of exogenous δ -ALA (39). Following growth in lactate medium supplemented with [¹⁴C] δ -ALA and immunoprecipitation, the heme-modified proteins were visualized by gel electrophoresis and autoradiography (Fig. 4). The results clearly demonstrated that some iso-1/CAT was heme modified, although to a lesser extent than the endogenous cytochrome *c*. Therefore, the apoiso-1 moiety of iso-1/CAT resembles the normal apocytochrome *c* in that at least a fraction was accessible to the cytochrome *c* heme lyase.

The final step in the cytochrome *c* import pathway is the localization of the holoprotein to the IMS, where it interacts with the cytochrome *c* reductase and oxidase complexes (13). Mitochondria were fractionated to determine the location of iso-1/CAT (Fig. 5). The crude mitochondria were lightly digested with trypsin to remove the bound but unprotected iso-1/CAT, and the mitoplasts were salt-extracted to release the IMS proteins. The mitoplast pellet was further fractionated into matrix and membranes. Immunoblots of each fraction were probed with antisera specific for cytochrome *c*, CAT, and the β -subunit of F₁-ATPase. The results showed that cytochrome *c* but not the fusion protein was enriched in the IMS, while neither was significantly

FIG. 4. Heme modification of iso-1/CAT. Extracts were prepared from LSR1 and YEpCYC1/cat transformants of LSR1 grown in DL-lactate medium containing the heme precursor [^{14}C]δ-ALA. Immunoprecipitates from 500 μg of extract, done with an excess of yeast cytochrome *c* and CAT antiserum, were collected with 150 μl of protein A-agarose and electrophoresed on a 15% SDS-polyacrylamide gel, and labeled proteins were detected by fluorography. Total refers to lanes containing 10 μg of total extract before immunoprecipitation; ppt refers to lanes containing immunoprecipitates. The positions of the heme-modified proteins cytochrome *c* (cyt *c*) and cytochrome *c*₁ (cyt *c*₁) are indicated on the left.

present in the matrix. Most of the fusion protein cofractionated with the membranes along with the F_1 -ATPase, a membrane marker protein. The most likely explanation for these results is that the apoiso-1 moiety can correctly initiate the import process, as evidenced by its accessibility to heme modification, but that the globular structure of the CAT domain is incompatible with translocation through the outer mitochondrial membrane.

Fusion proteins with a loosely structured reporter moiety can support respiratory growth. If the folded conformation of the reporter protein can block translocation, fusion proteins with less highly structured reporter molecules may be imported. Two approaches were taken to determine the role of reporter protein folding in import. First, a fusion protein was constructed with apo-CuMT as the reporter protein. CuMT

FIG. 5. Submitochondrial localization of iso-1/CAT. Mitochondria were isolated from a transformant of NA1 expressing iso-1/CAT and digested with trypsin (10 μg/ml) for 5 min at 30°C. The reaction was terminated with soybean trypsin inhibitor, and the mitochondria were subfractionated as described in Materials and Methods. Trypsinized mitochondria (Mito+Trypsin), mitoplasts (200 μg each), IMS, matrix (50 μg each), and total membranes (100 μg) were electrophoresed on a 15% SDS-polyacrylamide gel. These amounts reflect the relative recovery of total protein from each fraction and keep the control cytochrome *c* within the linear range of detection on the immunoblot. Proteins whose positions are indicated at the left were visualized by immunoblotting with a mixture of antisera to yeast cytochrome *c* (cyt *c*), CAT, and the F_1 -ATPase β-subunit.

TABLE 2. Respiratory growth analysis of transformants of the CYC1 deletion strain expressing either iso-1/CAT or iso-1/CuMT fusion protein

Strain	Vector	Fusion protein	CuSO ₄ (mM)	Generation time ^a (h)
NA1	None	None	0	5.5
N1B	None	None	0	NG
	YEpCYC1/cat	Iso-1/CAT	0	NG
	YEpCYC1/catΔ	Iso-1/CATΔ	0	5.5
	YEpCYC1/CUP1	Iso-1/CuMT	0	5.5
	YEpCYC1/CUP1	Iso-1/CuMT	0.5	18.0
	YEpCYC1	None	0	5.2
	YEpCYC1	None	2.0	5.9

^a Growth of host and transformant strains in medium containing DL-lactate as the sole carbon source was measured in Klett units at 30°C. NG, No growth.

is an ideal candidate because it is a small, loosely folded, monomeric protein that assumes a structured conformation upon binding copper (38). Second, if iso-1/CAT was targeted as a membrane-bound intermediate in the cytochrome *c* import pathway, unfolding the CAT domain in iso-1/CAT should allow its translocation to the IMS. A disruption of the CAT domain in iso-1/CAT was constructed by deletion of amino acid codons 72 to 106 between *MaeIII* sites in the CAT coding region. Cells expressing the resulting fusion protein, iso-1/CATΔ, were chloramphenicol sensitive (not shown), confirming the disruption of CAT structure.

Figure 1 depicts the hybrid gene cassettes constructed for the expression of iso-1/CuMT (YEpCYC1/CUP1) and iso-1-cytochrome *c* (YEpCYC1). To determine the biological function of the fusion proteins, transformants of CYC1 deletion strain N1B, expressing iso-1-cytochrome *c*, iso-1/CAT, iso-1/CATΔ, or iso-1/CuMT were tested for respiratory growth (Table 2). Surprisingly, the generation time of transformants expressing iso-1/CuMT and iso-1/CATΔ was identical to that of those expressing iso-1-cytochrome *c* and to the NA1 control strain when grown on lactate as a nonfermentable carbon source. This indicates that both fusion proteins were translocated to the IMS and functional in respiratory electron transfer. In contrast, transformants expressing iso-1/CAT were unable to grow on lactate (Table 2), a phenotype consistent with the association of this fusion protein with mitochondrial membranes and minimal localization to the IMS (Fig. 5).

The iso-1/CuMT fusion protein offered the opportunity to induce a folded conformation of the CuMT moiety in vivo by inclusion of copper in the growth medium (3, 38). A block in the translocation step of the import pathway by the copper-induced folding of the CuMT domain should be reflected in a reduced level of respiratory activity. Growth in the presence of copper sulfate was therefore measured as a reflection of respiratory function. The results showed a marked inhibition in the rate of iso-1/CuMT-dependent respiratory growth in the presence of 0.5 mM copper sulfate (Table 2). In contrast, iso-1-cytochrome *c*-dependent growth was unaffected by 2 mM copper sulfate, indicating that the growth inhibition was mediated through iso-1/CuMT and was not a toxic effect of copper on other cellular functions. Therefore, reporter proteins with a loosely folded conformation appear to be compatible with respiratory function.

Folding of the reporter protein inhibits translocation to the IMS without affecting mitochondrial membrane recognition and insertion. If iso-1/CuMT and iso-1/CATΔ were responsible for the observed respiratory growth, the intact fusion

FIG. 6. Protease protection and heme labeling of iso-1/CuMT and iso-1/CATΔ. Protease protection of iso-1/CuMT (A) and iso-1/CATΔ (B) in isolated mitochondria was visualized by immunoblotting with antibodies to yeast cytochrome *c* (A) or a mix of antibodies to yeast cytochrome *c* and bacterial CAT (B). Crude mitochondrial fractions were isolated from transformants of N1B expressing each fusion protein and treated with trypsin for the indicated times (in minutes) as described in the legend to Fig. 3. The first lane contained commercially prepared yeast cytochrome *c* as a standard. U, Undigested; +T, treatment of mitochondria with Triton X-100 prior to protease digestion. (C) Immunoprecipitation of total SDS-dissociated extracts (270 μg per lane) from the [14 C]δ-ALA labeling of strains LSR1 and N1B transformed with the indicated vector and expressing iso-1/CAT (YEPCYC1/cat), iso-1/CATΔ (YEPCYC1/cata), iso-1/CuMT (YEPCYC1/CUP1), or yeast cytochrome *c* (YEPCYC1) (cyt *c*).

proteins should be imported into mitochondria and heme modified. Mitochondria from transformants expressing these molecules were subjected to trypsin treatment, and the proteins were visualized by immunoblotting (Fig. 6). Iso-1/CuMT and iso-1/CATΔ proteins of the predicted sizes of 20.9 (Fig. 6A) and 35.5 (Fig. 6B) kDa, respectively, were present in mitochondrial fractions, and the majority remained stable to trypsin treatment. As with iso-1/CAT, treatment with nonionic detergent rendered both fusion proteins trypsin sensitive, confirming their mitochondrial uptake. No cytochrome *c* was detected in the undigested fraction, indicating that the fusion proteins themselves and not a degradation product were responsible for growth on lactate.

To directly determine whether iso-1/CuMT and iso-1/CATΔ were heme modified, transformants were grown in lactate medium supplemented with [14 C]δ-ALA (Fig. 6C). Proteins were immunoprecipitated from total-cell extracts and subjected to autoradiography following gel electrophoresis. The autoradiogram demonstrated that transformants of N1B expressing either iso-1/CuMT or iso-1/CATΔ had a heme-labeled protein of the expected size for each fusion protein. No heme-modified cytochrome *c*-sized molecule was detected in these cells, confirming that the fusion proteins and not degradation products were the biologically active species. The level of heme-modified fusion proteins was similar to the level observed for iso-1-cytochrome *c* expressed in the same strain from the vector YEPCYC1 (Fig. 6C, N1B). Because iso-1/CAT was unable to support growth on lactate, a direct comparison of the heme modification of iso-1/CAT and iso-1/CATΔ was made in LSR1, a strain expressing iso-1-cytochrome *c*. Consistent with its accumulation in the IMS, the level of heme attachment was higher for iso-1/CATΔ than for iso-1/CAT compared with the endogenous cytochrome *c* as an internal control (Fig. 6C, LSR1). These results establish that both iso-1/CuMT and iso-1/CATΔ were imported into the mitochondria, properly heme modified, and functional in supporting respiratory growth.

Heme modification of iso-1/CuMT was also measured in cells grown in various copper concentrations with [14 C]δ-ALA as the heme precursor (Fig. 7). This experiment was carried out in a strain expressing the endogenous iso-1-cytochrome *c* as an internal control. The results demonstrated a two- to threefold decrease in the heme modification of iso-1/CuMT (Fig. 7A) that was equal in magnitude to the inhibition of respiratory growth by copper (Table 2). This was not the result of heme lyase inhibition, because heme modification of the endogenous cytochrome *c* and of iso-1/CAT was unaffected by the addition of 2 mM copper. Despite the reduced level of heme modification, similar levels of iso-1/CuMT were targeted to mitochondria and were protease resistant regardless of the presence of copper in the growth medium (Fig. 7B), demonstrating that copper had no effect on the steady-state level or mitochondrial targeting of the iso-1/CuMT polypeptide. Therefore, the folded conformation of the reporter protein can influence the accumulation of biologically active fusion proteins in the IMS without affecting mitochondrial membrane recognition and insertion.

Heme-binding cysteines are required for translocation to the IMS but not for mitochondrial targeting of iso-1/CAT fusion proteins. Blocking heme attachment in vitro appears to arrest import at the intermediate stage of membrane insertion (8, 23, 24). The iso-1/CAT fusion protein was similarly blocked in translocation to the IMS because of the folded conformation of the CAT moiety. To determine whether the heme-binding cysteine residues were required to arrive at this intermediate stage in the import pathway in vivo, the cysteines at positions 19 and 22 in iso-1/CAT were converted to serines by site-directed mutagenesis to generate iso-1[Ser^{19,22}]/CAT. As shown in Fig. 8A, this fusion protein was indistinguishable from iso-1/CAT in its trypsin-resistant association with mitochondria in vivo. Similarly, pretreatment of mitochondria with nonionic detergent exposed the fusion protein to proteolysis. Therefore, covalent heme attachment through cysteines 19 and 22 was not required for mitochondrial targeting in vivo.

Iso-1[Ser^{19,22}]/CAT has the CAT moiety in its native conformation and the serine replacements at positions 19

FIG. 7. Effects of copper on heme modification and protease protection of iso-1/CuMT. (A) Extracts (500 μ g) prepared from LSR1 (no vector) and LSR1 transformants expressing iso-1/CAT (YEpCYC1/cat) or iso-1/CuMT (YEpCYC1/CUP1) were immunoprecipitated as described in the legend to Fig. 4. Cells were grown in DL-lactate medium containing the heme precursor [¹⁴C] δ -ALA and the concentration of CuSO₄ indicated above each lane. The positions of the fusion proteins and cytochrome *c* (cyt *c*) are marked at the left of the autoradiograph. (B) Protease treatment of crude mitochondrial fractions isolated from the LSR1 transformant expressing iso-1/CuMT and grown in DL-lactate medium in the absence (-) or presence (+) of 0.5 mM CuSO₄. Mitochondria were treated with trypsin as described in the legend to Fig. 3 for the indicated times (in minutes). +T refers to treatment of mitochondria with Triton X-100 prior to protease digestion.

and 22. Although neither of these features affects mitochondrial targeting, both may interfere with proper translocation of the fusion protein to the IMS. To determine the effects of the cysteine-to-serine mutations on an otherwise translocation-competent fusion protein, they were introduced into iso-1/CAT Δ to generate iso-1[Ser^{19, 22}]/CAT Δ . If this molecule is targeted to the outer mitochondrial membrane as expected, its failure to be translocated to the IMS should

FIG. 8. Targeting of iso-1[Ser^{19, 22}]/CAT and iso-1[Ser^{19, 22}]/CAT Δ to mitochondria. Crude mitochondrial fractions were isolated from NA1 transformant strains expressing either iso-1[Ser^{19, 22}]/CAT (A) or iso-1[Ser^{19, 22}]/CAT Δ (B) and treated over a time course (shown in minutes) with trypsin protease as described in the legend to Fig. 3. The position of each fusion protein is denoted at the left along with the endogenous yeast cytochrome *c* (cyt *c*). +T refers to treatment of mitochondria with Triton X-100 prior to protease digestion.

result in the degradation of the mutationally disrupted CAT domain by trypsin. As shown in Fig. 8B, a substantial amount of iso-1[Ser^{19, 22}]/CAT Δ was found in the untreated mitochondrial fraction, but all of this material was degraded by trypsin after the shortest time of digestion (15 min). This is in striking contrast to the trypsin-resistant association of iso-1/CAT Δ with mitochondria (Fig. 6B). These results establish that covalent heme attachment through cysteines 19 and 22 is required for the translocation step of the import pathway in vivo but not for mitochondrial membrane recognition and insertion. Moreover, the trypsin sensitivity of the CAT Δ moiety when translocation was blocked by preventing heme attachment provides direct evidence that the fusion proteins are targeted to the outer mitochondrial membrane, with the CAT domain exposed as a cytoplasmic tail.

DISCUSSION

Most proteins localized to the mitochondrial IMS are synthesized as larger precursor proteins and routed through the matrix with the cleavage of amino-terminal signal presequences (11). In contrast, cytochrome *c* is synthesized without an amino-terminal extension and localized to the IMS in the absence of proteolytic processing (13). It has been proposed that the initial step of the cytochrome *c* import pathway is the receptor-independent insertion of the apoprotein into the outer membrane (34). A mutational analysis with cytochrome *c* fusion proteins may be useful in delineating the structural features of the cytochrome *c* apoprotein required for the correct targeting to mitochondria in vivo. In this study we established that information in apoiso-1-cytochrome *c*, when expressed as part of a fusion protein, is capable of directing foreign reporter proteins to the mitochondria in vivo. Disruption of the folded conformation of the reporter molecule allows these fusion proteins to function efficiently in supporting respiratory growth, strongly indicating that they follow the cytochrome *c* import pathway.

The results demonstrated that (i) both iso-1/CAT and iso-1/CuMT can be stably expressed in yeast cells at steady-state levels equivalent to that of iso-1-cytochrome *c*, (ii) both were specifically targeted to mitochondria and protected from external trypsin treatment, and (iii) both were accessible to the cytochrome *c* heme lyase. In addition to these shared properties, iso-1/CuMT but not iso-1/CAT was able to support respiratory growth on lactate in an iso-1-cytochrome *c* deletion strain. Iso-1/CuMT was therefore localized to the IMS and had a structure compatible with efficient respiratory electron transfer. However, for both fusion proteins, factors that altered the folded conformation of their respective reporter proteins dramatically changed their behavior. Disruption of the CAT moiety in iso-1/CAT by deletion mutation to generate iso-1/CAT Δ resulted in its translocation to the IMS and efficient respiratory function. Conversely, induction of a folded conformation in iso-1/CuMT by including copper in the growth medium inhibited both heme attachment and ability to support respiratory growth. In contrast with previous observations (33), a free carboxy terminus was not required in either case for efficient import or catalytic function. The evidence strongly suggests that the differences in the behavior of the two fusion proteins lie in the membrane translocation step of the import pathway.

The behavior of iso-1/CAT and iso-1/CuMT may be best explained in light of the previously observed import properties of fusion proteins. For example, methotrexate stabiliza-

demonstrates that the requirement for heme attachment in vivo is restricted to the translocation step of the pathway. Moreover, the accessibility of the unfolded CATA domain to external proteolysis when translocation is blocked, as is the case for iso-1[Ser^{19, 22}]/CATA, provides further evidence that the fusion proteins are targeted to the outer mitochondrial membrane as intermediates in the cytochrome *c* import pathway.

In summary, apoiso-1-cytochrome *c* fusion proteins expressed in vivo faithfully mimic cytochrome *c* in several characteristics of its import pathway as defined in vitro. Most notable is the ability of iso-1/CuMT and iso-1/CATA to function efficiently in respiratory growth on lactate, establishing the compatibility of fusion protein structure with the biological function of cytochrome *c*. The folded conformation of the reporter protein moiety determines whether the fusion proteins are completely imported or arrested as membrane-associated intermediates in the import pathway. Fusion proteins with a folded reporter domain will be especially useful in defining structural features required for mitochondrial membrane targeting and insertion in vivo.

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