The Role of Hot13p and Redox Chemistry in the Mitochondrial TIM22 Import Pathway*

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The small Tim proteins in the mitochondrial intermembrane space participate in the TIM22 import pathway for assembly of the inner membrane. Assembly of the small TIM complexes requires the conserved “twin CX3C” motif that forms juxtapositional intramolecular disulfide bonds. Here we identify a new intramembrane space protein, Hot13p, as the first component of a pathway that mediates assembly of the small TIM complexes. The small Tim proteins require Hot13p for assembly into a 70-kDa complex in the intermembrane space. Once assembled the small TIM complexes escort hydrophobic inner membrane proteins en route to the TIM22 complex. The mechanism by which the small Tim proteins bind and release substrate is not understood, and we investigated the affect of oxidant/reductant treatment on the TIM22 import pathway. With organello import studies, oxidizing agents arrest the ADP/ATP carrier (AAC) bound to the Tim9p-Tim10p complex in the intermembrane space; this productive intermediate can be chased into the inner membrane upon subsequent treatment with reductant. Moreover, AAC import is markedly decreased by oxidant treatment in Δhot13 mitochondria and improved when Hot13p is overexpressed, suggesting Hot13p may function to remodel the small TIM complexes during import. Together these results suggest that the small TIM complexes have a specialized assembly pathway in the intermembrane space and that the local redox state of the TIM complexes may mediate translocation of inner membrane proteins.

Mitochondrial inner membrane biogenesis is an essential process in all eukaryotes. The Translocase of the Inner Membrane (TIM) 22 complex is dedicated to the insertion of nucle-

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The abbreviations used are: TIM, Translocase of Inner Membrane; TOM, Translocase of Outer Membrane; AAC, ADP/ATP carrier; DHFR, dihydrofolate reductase; DIOc3-, 3,3′-dihexylocarbocyanine iodide; ar-coded polytopic inner membrane proteins, including the mitochondrial carrier family and import components Tim22p and Tim23p (1–3). The 300-kDa membrane complex consists of a pore-forming subunit Tim22p, in addition to Tim18p and Tim54p, that plays an unknown function and a fraction of the small Tim proteins Tim9p, Tim10p, and Tim12p (1–3). Substrates are escorted from the Translocase of the Outer Membrane (TOM) complex to the TIM22 translocon by two distinct 70-kDa chaperone-like complexes in the intermembrane space: Tim9p-Tim10p and Tim8p-Tim13p (4–7).

Insertion into the inner membrane requires a membrane potential (ΔΨ), but the events by which a substrate is transported from the TOM complex to the inner membrane complex remain obscure. The carrier proteins and Tim23p are targeted to the Tom70p receptor on the outer membrane (7, 8) and then pass through the TOM complex as a loop (4, 5, 8). The small Tim proteins subsequently bind to hydrophobic domains in the substrates and chaperone them to the 300-kDa insertion complex (4, 5, 9). The substrate is transferred to the insertion complex and inserted into the inner membrane (10). Cryo-electron microscopy and reconstitution studies have shown that Tim22p forms a twin-pore translocase that is voltage-gated (10, 11).

The small Tim proteins contain the “twin CX3C” motif that is required for assembly of the 70-kDa complexes (12). The twin CX3C is conserved among all small Tim proteins (13, 14) and a mutation in the 4th cysteine leads to the X-linked disease deafness-dystonia syndrome/Mohr-Tranebjaerg syndrome, because the Tim8p-Tim13p complex fails to assemble (15). The specific function of the cysteine residues has been debated recently (12). One set of studies suggests that the motif is important for Zn2+ coordination in the small Tim proteins (16, 17), but a different set of studies suggests that the small Tim proteins form juxtapositional intramolecular disulfide bonds (4, 5, 18–20). Additional mitochondrial proteins, including the Rieske iron-sulfur protein and superoxide dismutase, also contain disulfide bonds (21, 22).

Pathways for the formation of disulfide bonds in the endoplasmic reticulum and bacterial periplasm have been well characterized (23), but Moss and colleagues (24) have demonstrated a complete pathway in the cytosol for disulfide bond insertion in the virion membrane proteins of poxvirus. Included in this pathway is an FAD-linked sulhydryl oxidase. The mitochondrial intermembrane space also contains the FAD-linked sulhydryl oxidase, Erv1p, suggesting an oxidative folding pathway might occur in this compartment (25).

HOT, Helper of Tim; MBS, m-maleimidobenzoyl-N-hydroxysuccinimide ester; θ, membrane potential; t-BOOH, t-butyl hydroperoxide; DTT, dithiothreitol; β-ME, β-mercaptoethanol; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.
Hot13p Mediates Assembly of the Small Tim Proteins

Fig. 1. Hot13p is a novel protein that localizes to the mitochondrial intermembrane space. A, radiolabeled Tim12p was imported into wild-type or Δhot13 mitochondria followed by cross-linking with 0.1 mM maleimido-benzoyl-N-hydroxy-succinimide ester (MBS). After quenching, the samples were separated by SDS-PAGE and analyzed by fluorography. The asterisk denotes a cross-link to a ∼15-kDa protein that is absent in Δhot13 mitochondria. As a standard (S), 5% of the Tim12p translation reaction added to mitochondria was included. B, Hot13p (YKL084W) contains 11 cysteine residues (shown in red). The tryptic peptide fragments identified by mass spectrometry (blue bars) covered 27% of the protein. C, GFP was integrated in-frame to the C terminus of HOT13 for localization studies. Mitochondria were visualized with the essential dye MitoTracker® Red CMXRos (right panel). Hot13p-GFP expression was visualized simultaneously (left panel), and the two images were superimposed (center panel). D, the Hot13p-HA mitochondria (M) were treated by osmotic shock to disrupt the OM (Mitoplast) in the presence and absence of 50 μg/ml proteinase K (PK) or 0.1% Triton X-100 (Triton) followed by centrifugation to separate the pellet (P) from the supernatant (S). Mitochondria (M) also were extracted with 0.1 M Na2CO3 (Carbonate) followed by centrifugation to separate the pellet (P) from supernatant (S). Equivalent amounts of protein were analyzed by SDS-PAGE and immunoblotting with polyclonal αTim12p, αTim10p, αcytochrome b6, αCpn10p, and αAAC. Hot13p-HA was identified with αHA. The asterisk denotes cross-reaction with porin. E, mitochondria containing Hot13p-HA were separated by SDS-PAGE in the presence (+) or absence (−) of DTT followed by immunoblotting with monoclonal αHA. Asterisks denote higher molecular mass products with Hot13p-HA.

EXPERIMENTAL PROCEDURES

Plasmids and Strains—Standard genetic techniques were used for growth, manipulation, and transformation of yeast strains (28–28). For expression of the recombinant Tim13p, TIM13 with a C-terminal 9× histidine tag was cloned into pET28a (Novagen). Expression was induced according to the manufacturer’s protocols (Novagen). HOT13 with a C-terminal hemagglutinin tag was cloned into the pRS420 series of vectors under the GPD1 promoter (PGPD) and PGK1 terminator (TPGK) for overexpression. A C-terminal HOT13-GFP fusion was created by PCR-based gene manipulation and integration (29).

Purification of Recombinant Tim13p-9XHis—Briefly, the overexpressed Tim13p-9XHis was purified on nickel-nitritrotriacetic acid metal affinity resin (QIAGEN) according to the manufacturer’s protocol under denaturing conditions. The urea-denatured Tim13p-9XHis was imported in chemical amounts for import and cross-linking studies in purified mitochondria.

Import of Radiolabeled Proteins into Isolated Mitochondria—Mitochondria were purified from lactate-grown yeast cells and assayed for in vitro protein import as described (25, 30). Proteins were synthesized in a rabbit reticulocyte lysate in the presence of [35S]methionine after in vitro transcription of the corresponding gene by SP6 polymerase. The reticulocyte lysate containing the radiolabeled precursor was incubated with isolated mitochondria at the indicated temperatures in import buffer (1 mg/ml bovine serum albumin, 0.6 M sorbitol, 150 mM KCl, 10 mM Hepes, pH 7.4). Where indicated, the potential across the mitochondrial inner membrane was dissipated with 1 μM valinomycin. Non-imported radiolabeled protein was removed by treatment with 100 μg/ml trypsin or 50 μg/ml protease K for 15–30 min on ice; trypsin...
was inhibited with 200 μg/ml soybean trypsin inhibitor and proteinase K with 1 mM phenylmethylsulfonyl fluoride, respectively. Where indicated, mitochondria and/or the in vitro translated precursors were pre-treated with oxidants (H₂O₂, t-BOOH, or oxidized glutathione) or reducing agents (DTT or β-ME) for the given time points on ice. For import reactions under oxidizing conditions followed by a chase with the redundant DTT, import reactions were performed in the presence of the oxidant t-BOOH followed by DTT addition to the import reaction after 1 min; the import reaction was mixed gently, and returned to the 25 °C water bath.

Cross-linking studies were performed as previously described (5). For immunoprecipitation assays, monoclonal anti-HA or anti-His antibody was coupled to protein A-Sepharose via a rabbit anti-mouse IgG antibody. Both bound proteins and coupled antibodies were removed by the addition of Laemmli sample buffer before SDS-PAGE analysis.

Microscopy Techniques—Hot13p-GFP in live cells was visualized on a Zeiss Axiovert 200 inverted fluorescence microscope utilizing a Fluorescein isothiocyanate filter cube set. Yeast cells expressing HOT13-GFP were first grown in rich medium with sucrose as a carbon source. The cells were washed in 1× phosphate-buffered saline buffer, counter-stained with 0.5 μM MitoTracker® Red CMXRos (Molecular Probes), and washed in 1× phosphate-buffered saline buffer three times to remove excess dye. The mitochondrial staining by MitoTracker® Red CMXRos was visualized with a Cy3 filter cube set.

Cova lent Modification of Intermembrane Space Proteins and Mass Spectrometry—A thiol-trapping approach similar to that used previously for the small Tim proteins (4, 5) was used to identify proteins coordinating disulfide bonds in the intermembrane space. Free sulfydryl groups were blocked with iodoacetamide followed by reduction of putative disulfide bonds with Tris(2-hydrochloride)phosphine hydrochloride and modification with a maleimide cross-linker (31). Proteins from the modified intermembrane space fraction were resolved by SDS-PAGE. Proteins with a molecular mass near 15–20 kDa were excised from the gel and subjected to in-gel digestion with trypsin. MALDI peptide fingerprint mass spectra were acquired by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS, Voyager DE-STR time-of-flight mass spectrometer from Applied Biosystems, Framingham, MA), using α-cyano-4-hydroxycinnamic acid (Sigma) as the matrix. Fingerprint mass spectra were analyzed with the ProteinProspector (version 4.0.5) analysis software.

Blue Native Gel Electrophoresis—Mitochondria (2.5 mg/ml) were solubilized in 20 mM K²-HEPES, pH 7.4, 50 mM NaCl, 10% glycerol, 2.5 mM MgCl₂, 1 mM EDTA, 0.16% n-dodecylmaltoside (Roche Applied Science) for 30 min on ice. Insoluble material was removed by centrifugation at 100,000 × g for 10 min, and the solubilized proteins were analyzed by blue native gel electrophoresis on a 6–16% linear polyacrylamide gradient.

Miscellaneous—Proteins were detected by immunoblotting using nickel-cassette- or polyvinylidene difluoride membranes and visualization of immune complexes with 125I-Protein A or via peroxidase-conjugated secondary antibodies followed by chemiluminescence. Protein concentration was assayed by the bicinchoninic acid method (Pierce) using bovine serum albumin as the standard. Where indicated, the total amount of mitochondrial protein is shown in micrograms. Quantification was performed by scanning laser densitometry with ImageQuaNT software (Amersham Biosciences).

![Figure 2](image1.png)

**FIG. 2.** Hot13p is cross-linked to Tim13p in *in organello* import assays. A, recombinant Tim13p-9XHis was imported into mitochondria expressing Hot13p-HA, followed by cross-linking (XL) and immunoprecipitation with monoclonal α-HA (IPα-HA) or an unrelated monoclonal antibody (Mock). Monoclonal α-HA antibody was coupled to Protein A-Sepharose via a rabbit anti-mouse IgG. Tim13p-9XHis was detected by immunoblotting with monoclonal α-His antibody, followed by a secondary rabbit anti-mouse IgG peroxidase-conjugated antibody and chemiluminescence. The arrow indicates the cross-linked Hot13p-HA-Tim13p-9XHis, and the asterisk denotes the position of the antibody heavy and light chains. The X denotes nonspecific bands recognized by the α-His antibody, and the • indicates a second cross-linked protein containing Hot13p-HA and Tim13p-9XHis. The standard (S) represents 5% of the Tim13p-9XHis recombinant protein that was imported into Hot13p-HA mitochondria. B, Tim13p-9XHis was imported as in A followed by purification with Ni²⁺-agarose and immunoblotting with α-HA (left panel) and immunoprecipitation with α-HA followed by immunoblotting with α-His (right panel). The symbol nomenclature associated with the immunoblot is identical to that in A.

![Figure 3](image2.png)

**FIG. 3.** The TIM22 import complexes are not assembled correctly in Δhot13 mitochondria. A, mitochondrial proteins (50, 100, and 150 μg) from the parental (WT) and Δhot13 strain were separated by SDS-PAGE, followed by immunoblot analysis with antisera against OM proteins porin, Tom40p, and Tom70p; inner membrane proteins the F₀ subunit of the ATPase, AAC, Tom23p, Tim54p, and Tim22p; intermembrane space proteins cytochrome b₅ (cyt b₅), Tim8p, Tim13p, Tim23p, Tim12p, and Tim10p; and matrix proteins aconitate, mHsp70, and Hsp60. The asterisk denotes a nonspecific band recognized by the Tim22p antibody. B, as in A mitochondrial proteins were separated on a 6–16% blue native gel followed by immunoblotting with antisera for Tim8p, Tim9p, Tim10p, Tim12p, and Tim22p, the core cytochrome b₅ complex, and subunit F₁-β of the ATPase.
RESULTS

Hot13p Mediates Assembly of the Small Tim Proteins

The assembly pathway of the small Tim proteins was characterized by in organello import studies and cross-linking (Fig. 1A). A thiol-trapping approach similar to that used previously for the small Tim proteins in combination with mass spectrometry was used to identify proteins in the intermembrane space that might form disulfide linkages with a molecular mass near 15 kDa (4, 5). The novel protein coded by the yeast open reading frame YKL084W, referred to as Helper of Tim (HOT) 13, HOT13, was identified with a predicted molecular mass of 13.5 kDa and with 11 cysteine residues (Fig. 1B). Hot13p is conserved among yeast and prokaryotes and is a member of the zf-CHY domain family (32), which has strong similarity to a RING finger domain. Higher eukaryotes contain larger proteins with the zf-CHY domain. Proteins containing RING finger motifs coordinate two Zn$^{2+}$ ions, and these motifs are thought to facilitate protein-protein interactions (33).

Hot13p-GFP co-localized with MitoTracker when visualized by fluorescence microscopy (Fig. 1C). In addition, Hot13p-HA localized to the intermembrane space in isolated mitochondria (Fig. 1D). When the outer membrane was disrupted by osmotic shock, Hot13p-HA remained associated with the mitochondrial membranes, but protease addition resulted in degradation of both Tim12p and Hot13p, confirming localization within the intermembrane space either in association with the outer or inner membrane. In addition, Hot13p was a peripheral membrane protein, because it was released by carbonic acid extraction, in contrast to an integral inner membrane protein, the ADP/ATP carrier (AAC). Hot13p-HA also formed higher molecular mass complexes using non-reducing denaturing electrophoresis (Fig. 1E), suggesting that the cysteine residues may form disulfide bonds. HOT13 was not essential for viability, but strains deleted for HOT13 showed a 30% decrease in growth in the presence of the oxidant t-butyl hydroperoxide (t-BOOH).

We confirmed that Hot13p was the cross-linked protein that bound to the small Tim proteins by performing import assays into mitochondria lacking Hot13p (Δhot13; Fig. 1A) and mitochondria containing Hot13p with a C-terminal hemagglutinin tag (Hot13p-HA; Fig. 2). The cross-linked product, denoted with an asterisk, was not detected in Δhot13 mitochondria (Fig. 1A). In the Hot13p-HA mitochondria, chemical amounts of Tim13p-9XHis were imported followed by cross-linking and immunoprecipitation with monoclonal anti-HA antisera (Fig. 2A and B) or affinity purification with Ni$^{2+}$-agarose (Fig. 2B). Monoclonal anti-HA was coupled to protein A-Sepharose via rabbit anti-mouse IgG. Under reciprocal purification schemes, Hot13p-HA was cross-linked to Tim13p-9XHis (indicated by the arrow in Fig. 2). Specifically, imported Tim13p-9XHis was cross-linked to two bands marked with an arrow (representing the Hot13p-HA-Tim13p-9XHis cross-link; Fig. 2A, lanes 3 and 4 and Fig. 2B, lanes 2 and 4) and a circle. The circle (Fig. 2A, lane 3; Fig. 2B, lane 2) presumably marks a second cross-linked product between Hot13p-HA and Tim13p-9XHis, because this cross-linked product was purified with Ni$^{2+}$-agarose (Fig. 2B, lane 2) and detected with anti-HA antibody; cross-linking potentially interfered with the interaction between the monoclonal HA antibody and Hot13p-HA resulting in a failed immunoprecipitation for this cross-linked product (Fig. 2A, lanes 3

![Figure 4](https://example.com/figure4.png)

**Fig. 4.** Hot13p facilitates assembly of the small TIM complexes. A, radiolabeled Tim9p and Tim10p were synthesized in vitro and precipitated with ammonium sulfate. The precipitated precursor was solubilized in 7 M urea and imported into wild-type and Δhot13 mitochondria at $25^\circ$C in the presence and absence of a membrane potential ($\Delta V$). Samples were treated with protease to remove non-imported precursor. B, Tim10p was imported in the presence of a non-imported precursor and assembly of imported Tim10p with endogenous Tim9p was analyzed by separation on a 6–16% blue native gel. The upper arrow indicates the 70-kDa assembled Tim9p-Tim10p complex, and the lower arrow indicates the unassembled Tim10p monomer. 10% of the imported Tim10p is shown as the standard (Std).

| Table 1 |
| List of strains used in this study |

| Strain          | Allele                                         | Source       |
|-----------------|-----------------------------------------------|--------------|
| WT              | MATa ade8 his3 leu2 trp1 ura3                  | (44)         |
| Δtim8Δtim13     | MATa ade8 his3 leu2 trp1 ura3 tim8::URA3 tim13::KANMX2 | (45)         |
| Tim9–3          | MATa ade8 his3 leu2 trp1 ura3 tim9::TRP1 tim9–3::HIS3 | (28)         |
| Tim10–1         | MATa ade8 his3 leu2 trp1 ura3 tim10::HIS3 tim10–3::LEU2 | This study   |
| Tim12–1         | MATa ade8 his3 leu2 trp1 ura3 tim12::HIS3 tim12–17::LEU2 | This study   |
| Δtim18          | MATa ade8 his3 leu2 trp1 ura3 tim18::KANMX2    | This study   |
| Tim22–19        | MATa ade8 his3 leu2 trp1 ura3 tim22::HIS3 tim22–19::LEU2 | This study   |
| Δtim54          | MATa ade8 his3 leu2 trp1 ura3 tim54::HIS3      | This study   |
| Tim23ts         | tim23::2                                       | (46)         |
| Δhot13          | MATa his3 leu2 met15 ura3 ykl084w::KANMX2      | Resgen®      |
| Hot13-GFP       | MATa ade8 his3 leu2 trp1 ura3 HOTT13GFP::HIS3  | This study   |
| ↑Hot13p         | MATa ade8 his3 leu2 trp1 ura3 [pRS423-PGPD-HOT13HA-TPGK] | This study   |
Hot13p Mediates Assembly of the Small Tim Proteins

versus 5). For immunoblotting, monoclonal anti-HA and anti-His antibodies were used followed by detection with anti-mouse-horseradish peroxidase-conjugated secondary antibody and chemiluminescence; the heavy and light chain antibodies detected in the immunoprecipitation lanes are marked with an asterisk and are shown in the immunoprecipitation control with an unrelated monoclonal antibody (denoted “mock,” Fig. 2A, lane 4). Hot13p thus binds to the small Tim proteins during import.

Hot13p Mediates Assembly of the Small TIM Complexes—How might Hot13p affect the TIM22 pathway? We analyzed the steady-state levels of proteins in mitochondria deleted for Hot13p using immunoblot analysis (Fig. 3A). Specifically, the abundance of the small Tim proteins, Tim8p, Tim9p, Tim10p, Tim12p, and Tim13p as well as Tim23p was decreased ~80–90% in Δhot13 mitochondria. The abundance of Tim23p also was slightly decreased in Δhot13 mitochondria, but this may be because Tim23p is dependent upon the TIM22 pathway for import (5, 34). The steady-state levels of control proteins in the outer membrane (porin, Tom40p, and Tom70p), intermembrane space (cytochrome b$_{2}$), inner membrane (F1α-ATPase and AAC), and matrix (aconitase, Hsp70, and Hsp60) were similar between Δhot13 and WT mitochondria. In addition, the assembly of the TIM22 complexes was examined by blue native gel electrophoresis (Fig. 3B). Mitochondria deleted for Hot13p lacked the 70-kDa Tim8p-Tim13p complex and had significantly reduced amounts of the 70-kDa Tim9p-Tim10p complex and inner membrane 300-kDa TIM22 complex. However, complex III and complex V of the respiratory chain assembled as expected, indicating pleiotropic defects in the integrity of the mitochondrial inner membrane were not present. Because the TIM22 components do not assemble correctly, they are degraded, presumably by mitochondrial proteases as we have previously shown with mutant Tim8p in deafness-dystonia syndrome (15). Further, mitochondrial membrane potential measurements showed that Δhot13 mitochondria and wild-type mitochondria potentials were similar. Thus, deletion of HOT13 seems to affect assembly of complexes in the TIM22 import pathway specifically.

Previous studies by Tokatlidis and colleagues (18, 19, 35) have shown that the Tim9p-Tim10p complex can be reconstituted in mitochondria lacking the endogenous Tim9p-Tim10p complex. Specifically, radiolabeled Tim9p or Tim10p, when imported into temperature-sensitive tim9 or tim10 mitochondria that lack the endogenous Tim9p-Tim10p complex, respectively, assemble into a 70-kDa complex. Unassembled endogenous partner protein is available associated with the membrane (35). When similar experiments were performed using recombinant Tim9p or Tim10p, the assembled Tim9p-Tim10p subsequently restored import of carrier proteins, indicating that an active Tim9p-Tim10p complex can assemble (35). We therefore investigated whether Hot13p mediated assembly of the 70-kDa complexes by investigating the assembly of the 70-kDa complexes in Δhot13 mitochondria using in vitro import assays followed by blue-native gel electrophoresis to monitor complex assembly (Fig. 4). Import of the small Tim proteins, Tim9p and Tim10p, was reduced by 35% into isolated Δhot13 mitochondria (Fig. 4A), but Δhot13 mitochondria were competent to import Tim9p and Tim10p. Assembly was then monitored by blue-native gel electrophoresis (Fig. 4B). A fraction of the imported Tim10p assembled with available, endogenous Tim9p to form a 70-kDa complex in wild-type mitochondria; however, the 70-kDa Tim9p-Tim10p complex failed to assemble in mitochondria lacking Hot13p. Similar results were obtained with import of the Tim9p monomer. When Tim10p was imported under oxidizing conditions in the presence of t-BOOH in wild-type mitochondria, the imported Tim10p also assembled with available Tim9p into a 70-kDa complex. These experiments thus suggest that Hot13p is required for the assembly of the 70-kDa Tim9p-Tim10p complex in the intermembrane space.

AAC Import Is Affected by Redox Treatment to Mitochondria—The events in which the small TIM complexes bind and transfer substrate prior to the insertion into the inner membrane complex are vague. Because the small TIM complexes contain disulfide linkages, we further investigated whether the import of substrates might be affected by the redox state of the small

**Fig. 5.** Protein import into the mitochondrial inner membrane is redox sensitive. A, yeast strains defective in the TIM22 import pathway are sensitive to oxidant. Haploid strains were grown at 25°C in YPD supplemented with 1 mM tert-butyl hydroperoxide (t-BOOH) and growth rates were analyzed at A$_{600}$. Growth rates for all strains were similar to the parental strain (WT) in YPD media at 25°C. The log of the growth was plotted versus time for the following strains: WT (GA74) (solid line) (36), tim9-3 (dashed line) (42), tim12-1 (○) (36), Δtim18 (□) (43), tim22-19 (□) (34), and Δtim54 (○). B, radiolabeled TIM23 subunit pSU9-DHFR and AAC were synthesized in vitro and imported in the presence of t-BOOH into WT mitochondria that were pretreated with t-BOOH for 15 min prior to import. At the indicated time points, equal aliquots were removed, and samples were treated with protease to remove non-imported precursor. AAC samples were extracted with carbonate to remove any precursor that was not inserted into the inner membrane. 100% was set as the amount of precursor imported into untreated mitochondria after 4 min. STD, 20% of the radioactive precursor added to the assay; p, precursor; m, mature. C, the TIM22 substrate Tim23p was imported as described for AAC in B except mitochondria were pretreated with 2.0 mM t-BOOH. No treatment (solid line); t-BOOH treatment (dashed line). D, the TIM23 substrate cytochrome b$_{2}$-DHFR was imported as described for pSU9-DHFR in B except mitochondria were pretreated with 2.0 mM t-BOOH.
Tim proteins. We previously generated a collection of yeast strains containing conditional alleles for the essential import components or deletions of the non-essential import genes (Table 1). These strains grew at a similar rate to the parental strain on YPD media at 25 °C (28, 34), which is the permissive temperature for the conditional mutants. The collection was tested for growth sensitivity in YPD supplemented with 1 mM t-BOOH as in Fig. 2B, D, WT mitochondria were pre-treated for 15 min with 2 mM t-BOOH at 25 °C to arrest AAC import as in Fig. 2B. AAC precursor (+ precursor) was added and, after 1 min, the import reaction was split and the incubation (chase) was continued in the presence of t-BOOH or 4 mM DTT. Samples were treated with protease and carbonate as in Fig. 2B, E, the membrane potential ΔΨ of wild-type mitochondria untreated (left panel) and treated with 2.0 mM t-BOOH (right panel) as in Fig. 2B was assessed at 25 °C using the potential-sensitive dye DIOC₆. Mitochondria sequestered dye in a membrane potential manner that was released upon the addition of the uncoupler valinomycin. ΔΨ was assessed by the difference in fluorescence before and after the addition of the valinomycin (Δ, indicated by the double-headed arrow). F, radiolabeled AAC was imported into mitochondria deleted for Hot13p (Δhot13) or overexpressing Hot13p-HA/ΔHot13p as in C in the absence or presence of 2 mM t-BOOH. 100% was set as the amount imported into untreated wild-type mitochondria in the presence of a membrane potential.

We have previously used a thiol-trapping approach to show that in the presence of reductant the small Tim proteins do not form disulfide bonds but in the presence of oxidant the disulfides are restored (4, 5). We therefore investigated the influence of the oxidant t-BOOH and reductant β-ME on the in organello import of the radiolabeled substrates AAC and Tim23p, which utilize the TIM22 import pathway, and the synthetic precursors pSu9-DHFR and cytochrome b₅-DHFR, which utilize the TIM23 translocon (Fig. 5, B–D). Radiolabeled AAC was imported into wild-type mitochondria preincubated with increasing amounts of t-BOOH followed by carbonate extraction to confirm insertion into the inner membrane (Fig. 5C). The addition of 2 mM t-BOOH resulted in a 75 and 50% decrease in the import of AAC and Tim23p, respectively, but showed little effect on the import of matrix-localized pSu9-DHFR and intermembrane space-localized cytochrome b₅-DHFR (Fig. 5, B–D).

Oxidant H₂O₂ and oxidized glutathione also specifically decreased the import rate of AAC. AAC import into isolated mouse liver mitochondria pre-treated with t-BOOH showed a similar defect as in our yeast model system. Thus, oxidant-reductant treatment affects the TIM22 translocation system in yeast and mammalian mitochondria.
The import of the carriers has been well characterized and can be separated into five distinct stages (4, 8). We tested if translocation of AAC across the outer membrane was affected by oxidant by performing import in the absence of a membrane potential followed by protease treatment to remove non-imported precursor (Fig. 6A); the carbonate extraction step was omitted so AAC that was arrested in the intermembrane space could be detected by fluorography. The rate of AAC translocation across the outer membrane was not affected (Fig. 6A). Instead, the translocation intermediate was arrested in the intermembrane space in the presence of 2 mM t-BOOH. The arrested AAC intermediate was bound to the Tim9p-Tim10p complex, because cross-linked AAC was immunoprecipitated with antibodies against Tim9p and Tim10p (Fig. 6B). As a control, pretreatment of the precursor with oxidant or reductant prior to incubation with isolated mitochondria did not affect the import competency of the AAC precursor (Fig. 6C). Treatment with t-BOOH therefore results in an arrest at stage IIIb in the intermembrane space (4), which is similar to the translocation arrest previously observed in the tim12-1 conditional mutant (36).

The arrested translocation intermediate was productive, because import into the inner membrane was chased by the addition of DTT (Fig. 6D). AAC was imported in 2 mM t-BOOH as in Fig. 5B, except that following the 1-min time point, half of the reaction was chased with 4 mM DTT. All samples were treated with carbonate to confirm insertion into the inner membrane. The DTT chase resulted in the completion of AAC insertion into the inner membrane. Thus, the arrested import intermediate is productive and is not aggregated in the intermembrane space. We tested the ability of oxidized mitochondria to take up the dye 3,3’-dihexyloxacarbocyanine iodide (DiOC6) to ensure that the addition of t-BOOH did not have any secondary effects on membrane potential (37). Treatment with t-BOOH resulted in a slightly higher membrane potential in wild-type mitochondria (Fig. 6E). Taken together, these experiments suggest that import into the inner membrane via the TIM22 pathway may be mediated by changes in the redox state of the small Tim proteins.

The import of AAC in the presence of oxidant was tested in mitochondria lacking Hot13p (Δhot13) or mitochondria overexpressing Hot13p (↑Hot13p, Fig. 6F). AAC import was decreased in the presence of t-BOOH in Δhot13 mitochondria in comparison to wild-type; but when Hot13p was overexpressed, AAC import was enhanced in the presence of t-BOOH compared with that shown in Fig. 5B. Hot13p thus seemingly facilitates AAC import in the presence of t-BOOH, perhaps by remodeling the small TIM complexes.

**DISCUSSION**

The function of the twin CX_2C motif of the small Tim proteins has been a debated topic. A series of studies has shown that the small Tim proteins bind Zn^{2+} (16, 17, 38). Yet, alternative studies have shown that the small Tim proteins form intramolecular disulfide bonds (4, 5, 18–20). Tokatidis and colleagues (19) have examined the assembly of the Tim9p-Tim10p complex in detail and suggest that the small Tim proteins can bind zinc, but insertion of intramolecular disulfide bonds is requisite for assembly of the complex.

The assembly process of the TIM translocons is not well understood. We therefore investigated whether additional proteins might be required for the assembly of the small TIM complexes and identified the intermembrane space protein Hot13p. Because Hot13p has 11 cysteine residues and forms higher molecular mass species when resolved under non-reducing SDS-PAGE, it might have a role in thiol bond formation. Additionally, the Hot13p sequence predicts a RING structure that might coordinate Zn^{2+} and may facilitate protein-protein interactions. Although Hot13p is involved in the assembly of the small TIM complexes, Hot13p is not essential for viability; this is not unexpected, because previous studies by our laboratory have shown that the essential function associated with Tim9p and Tim10p is with Tim22p at the inner membrane and the 70-kDa complexes merely enhance movement of precursors across the intermembrane space (39). Additionally, because our Tim22p antibody is not very robust, the abundance of Tim22p may be underestimated (34).

The redox state of the intermembrane space is thought to be similar to the cytoplasm because of the free exchange of small molecules through porin in the outer membrane. Although this compartment is predicted to be reducing, the small Tim proteins, Sod1p (21), and Rip1p (22), have disulfide linkages. Therefore we investigated whether the import of Tim22 substrates might be affected by changes in the local redox state of the small TIM complexes by altering the redox state of the organelle. In import studies in the presence of oxidant, an AAC translocation intermediate bound to Tim9p-Tim10p arrested in the intermembrane space. Subsequent treatment with reductant chased the AAC intermediate into the inner membrane. We can not show that Hot13p affects AAC import via changes in the assembly state of the Tim9p-Tim10p complex; however, import of AAC into Δhot13 mitochondria was decreased in comparison to wild-type mitochondria and overexpression of Hot13p improved AAC import in the presence of oxidant.

We propose a model in which Hot13p mediates assembly and possibly recycling of the small TIM complexes (Fig. 7). Hot13p directly interacts with the newly imported small Tim proteins to assemble them into a 70-kDa complex. Under local oxidizing conditions, the small TIM complexes can bind to substrate, and the precursor is arrested and can not reach the inner membrane. As shown by treatment with reductant in the AAC import assays, the small TIM complexes might subsequently release substrates through conformational changes induced by the reduction of the disulfide bonds, resulting in transport of the substrate to the TIM22 insertion complex at the inner membrane. Because the abundance of Hot13p influenced the potency of the oxidant effect on AAC import, Hot13p might also

**Fig. 7. Model for the role of Hot13p and redox chemistry in the TIM22 import pathway.** Hot13p is a peripheral inner membrane protein in the intermembrane space. Newly imported Tim proteins interact with Hot13p and assemble into soluble 70-kDa complexes in the intermembrane space. After crossing the TOM complex, the inner membrane substrates are escorted across the intermembrane space to the TIM22 membrane complex. Under oxidizing conditions, the substrates remain bound to the small TIM complexes in the intermembrane space. Subsequent treatment with reductant results in release of the substrate from the small TIM complexes and insertion into the inner membrane. Hot13p may facilitate the release of substrate from the small TIM complexes and potential recycling of the small TIM complex for another round of import (denoted with the “?” symbol).
play additional roles in the TIM22 import pathway, possibly in reassembly and recycling of the small TIM complexes for another round of import. Under physiological conditions, Hot13p most likely works with other proteins in an oxidative folding pathway. An example of such a pathway is present in the cytosol in which the porphyrin has a redox pathway for inserting disulfide bonds in the virion membrane (24); this pathway consists of a virally coded sulfhydryl oxidase and a thioredoxin-like protein. It is possible that the mitochondrial intermembrane space may possess a similar pathway with the resident protein Erv1p, a sulfhydryl oxidase, and a thioredoxin system.

These data suggest that the import of TIM22 substrates is redox-regulated and that a new pathway in the mitochondrial intermembrane space facilitates the assembly of the small TIM proteins. Future studies are aimed at identifying additional players in this pathway and determining the mechanistic role of redox in the import of inner membrane proteins.

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