The qualitative relationship between preprotein translocases in the mitochondrial outer and inner membranes was determined by both a functional analysis and a determination of characteristic components of the translocases. Translocation contact sites of isolated mitochondria were saturated with intermediates of a matrix-targeted precursor of the F_{1}-ATPase (pF_{1}β), and import of preproteins into the different mitochondrial subcompartments was monitored. A strong inhibition (75–95%) was observed for preproteins with an N-terminal matrix targeting signal, indicating that a significant portion of the contact sites was blocked by accumulated F_{1}β. Insertion of preproteins into the outer membrane and import into the intermembrane space of preproteins without matrix targeting signals was inhibited by about 45%, indicating that functional outer membrane translocases were available despite saturation of contact sites. Similarly, import of members of the mitochondrial carrier family into the inner membrane was only partly inhibited (40–50%), demonstrating that functional Tim22 translocases are available to cooperate with the Tom machinery in import of matrix proteins. The stoichiometry of Tom40, Tim23, and Tim22 in mitochondria was determined to be 5:1:0.22. We conclude that translocases of the outer membrane are present in excess over translocases of the inner membrane.

Import of nuclear encoded mitochondrial preproteins into the mitochondria is a multistep process (1). To reach the mitochondrial matrix, precursor proteins are translocated across the mitochondrial outer and inner membranes. Biochemical and morphological studies revealed that protein import into the matrix proceeds through proteinaceous channels at so-called translocation contact sites (2–7). In the electron microscope, regions of close proximity of the mitochondrial outer membrane to the inner membrane have been observed (5). These areas are particularly conspicuous in mitochondria in which the matrix space has been shrunk and the inner membrane is pulled away from the outer membrane. They are often referred to as morphological contact sites. Contact sites may be the preferred location where translocation contacts of the Tom and Tim complexes are formed, but this issue has not been finally settled. With biochemical means, contact sites between Tom and Tim complexes were only detected in the presence of a translocating polypeptide chain (6, 7). Components of the Tom machinery and Tim23, Tim17, as well as mt-Hsp70 and Tim44 were found in such super-complexes.

For import of members of the mitochondrial carrier family, such as the ADP/ATP carrier, the Tom machinery cooperates with Tim22 (8). This pathway does not require functional Tim23 and mt-Hsp70 (8). The translocation machinery of the outer membrane can also act independent of the translocases of the inner membrane (9). Similarly, in mitoplast preparations, preproteins are imported directly via Tim23 into the matrix (10). This raises the question of how the Tom machinery interacts with the inner membrane translocases Tim23/Tim17 and Tim22 and how the translocases are related stoichiometrically.

In this study, chemical amounts of a matrix-targeted precursor protein were incubated with isolated mitochondria under conditions optimal for import. The effect of accumulation of this precursor in a membrane-spanning fashion on the subsequent import of preproteins into the different mitochondrial subcompartments was analyzed. In this situation, import of preproteins into the matrix is blocked. The results demonstrate that translocation contact sites for import of preproteins into the matrix can be saturated. However, functional Tom machinery is still available for import of proteins into the outer membrane and the intermembrane space, suggesting that Tom complexes are in excess over Tim23/Tim17 complexes. Tom machinery is also available for cooperation with Tim22 to facilitate import of members of the mitochondrial carrier family into the inner membrane. A quantification of the mitochondrial content of Tom40, Tim23, and Tim22 supports the notion that translocases of the mitochondrial outer membrane are present in excess over inner membrane import machinery. The data suggest that a preprotein associates with the Tom complex and then recruits the appropriate machinery required for its sorting to mitochondrial subcompartments.

**EXPERIMENTAL PROCEDURES**

Preparation of Radiolabeled pF_{1}β—The c-DNA encoding the precursor of the β-subunit of the F_{1}F_{0}ATPase (pF_{1}β) from *Neurospora crassa* (11) was modified as follows. An NdeI restriction site was introduced in front of the start codon by oligonucleotide-directed site-specific mutagenesis using the polymerase chain reaction. The resulting DNA fragment was ligated into pJLA503 (12), which was cleaved with NdeI and EcoRI. The plasmid was used for transformation of *Escherichia coli* DH1.

A single colony of the transformed *E. coli* strain was grown overnight at 28 °C in M9 minimal medium (0.4% (w/v) glucose, 0.5% NaCl, 6% Na_{2}HPO_{4}, 3% KH_{2}PO_{4}, 1% NH_{4}Cl, 1 mM MgCl_{2}, 1 mM CaCl_{2}, 1 mg/liter thiamin) containing 0.4% (w/v) casamino acids and 100 mg/liter ampicillin. This culture was diluted to an 

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of each amino acid except methionine and cysteine), prewarmed to 42 °C, and supplied with 50 μCi of [35S]sulfate at a final concentration of 10 μM. Incubation at 42 °C was continued for 2 h.

Cells were reisolated in 2% of the original volume in buffer A (25% (w/v) sucrose, 50 mM Tris-HCl, pH 8.0), 1 mg/ml lysozyme and incubated for 30 min at room temperature. Then, the volume was adjusted to 20% of the original culture volume with buffer A supplied with 25 mM EDTA, 1% (w/v) Triton X-100, 10 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. The cells were sonicated in a Branson sonifier (3 × 10 pulses, 40% duty, maximal setting), and inclusion bodies of pF1β were pelleted by centrifugation for 30 min at 40,000 × g. The pellet was resuspended by sonication in buffer B containing 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100 and centrifuged as above. Finally, the pellet was washed twice as above in buffer B lacking Triton X-100. The final pellet was solubilized in buffer C (7 M urea, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 30 mM histidine, pH 6.0). The pF1β protein was purified from these inclusion bodies by chromatography on DEAE-Sephacel equilibrated with buffer C. The column was developed with a gradient of buffer C from 0 mM to 200 mM NaCl. The protein eluted at 50 mM NaCl.

In Vitro Synthesis of Precursor Proteins and Import into Mitochondria—Precursor proteins were synthesized by coupled transcription/translation in reticulocyte lysate in the presence of [35S]methionine (Amersham) as described (15). Wild-type Saccharomyces cerevisiae strain MC3:YPGAL0TC (14) was grown overnight in lactate medium, cells were harvested at A540 = 1–1.5, and mitochondria were isolated (15).

Import reactions were carried out at 25 °C in 200 μl of import buffer (600 mM sorbitol, 1 mg/ml bovine serum albumin, 50 mM KCl, 10 mM MgCl2, 2.5 mM EDTA, 2 mM KH2PO4, 2 mM ATP, 5 mM NADH, 50 mM HEPES, pH 7.2) containing 20 μg of mitochondria and 5–10% reticulocyte lysate with radiolabeled precursor. Where indicated, the membrane potential was dissipated with 50 μM carbonyl cyanide m-chlorophenylhydrazone. Import was assayed by treating the mitochondria with 100 μg/ml protease K for 15 min on ice, and samples were analyzed by SDS-PAGE and fluorography.

Immunoprecipitation—Yeast cells were grown overnight at 30 °C in the presence of [35S]sulfate (300 mCi/mol). Mitochondria were prepared, and aliquots corresponding to 50 μg of protein were solubilized in TBS containing 0.05% SDS and 0.5% Triton X-100. After a clarifying spin, the supernatant was subjected to immunoprecipitation with anti-Tom40 IgG (10 μg), with affinity-purified anti-Tim23 IgG (0.5 μg) and affinity-purified anti-Tim17 IgG (0.5 μg), respectively. Immunoprecipitates were analyzed by SDS-PAGE and quantified with a phosphorimaging system.

RESULTS

Accumulation of Translocation Intermediates in a Membrane-spanning Fashion—The precursor of the β-subunit of the mitochondrial ATP synthase (pF1β) (11) (0.1 μg) was diluted out of urea into an import assay containing S. cerevisiae mitochondria (30 μg). When the import reaction was carried out for 30 min at 10 °C, the precursor was efficiently processed to the mature form; however, only about one-third of the processed species was resistant to externally added protease K (PK) (Fig. 1). This indicates that the kinetics of translocation of the polypeptide chain were slow at low temperature, and a significant fraction of the protein was present in a membrane-spanning configuration, with the N terminus processed in the matrix and the C terminus on the outside and accessible to externally added PK. To saturate the mitochondrial import sites with membrane-spanning translocation intermediates, import reactions with pF1β were performed at 10 °C, and antibodies directed against the mature F1β (mF1β) were added. The amount of precursor recovered with the mitochondrial pellet increased with increasing amounts of anti-F1β IgG. This may reflect cross-linking of the preprotein by the antibody. In the presence of 10–100 μg of anti-F1β IgG, about 30 ng of F1β were processed. This was slightly more than without IgG, sug-

1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; PK, proteinase K.

FIG. 1. Accumulation of translocation intermediates in contact sites. Purified [35S]-labeled pF1β protein (0.1 μg) was incubated with energized yeast mitochondria (30 μg) for 30 min at 10 °C in the presence of the indicated amounts of IgG against the F1β-IgG. Mitochondria were reisolated after the import reaction, and one-half was treated with 100 μg/ml PK. The samples were analyzed by SDS-PAGE, and fluorography for 12 h (upper panel) and 48 h (lower panel). p, precursor, m, mature protein.
Second stage import of the outer membrane protein porin and of cytochrome c heme lyase into the intermembrane space was inhibited by about 40% (Fig. 2). Both proteins are synthesized without a cleavable presequence and imported via the Tom complex of the outer membrane (10, 20). These data suggest that despite saturation of translocation contact sites for protein import into the matrix, functional Tom machinery is available for import of proteins into the outer membrane and the intermembrane space.

The integral inner membrane proteins ADP/ATP carrier (AAC) and inorganic phosphate carrier (PiC) are synthesized without a cleavable presequence. Their import into the inner membrane requires the membrane potential ΔΨ and is facilitated by Tim22 in a reaction that does not need functional Tim23 and mt-Hsp70 (8). When F1β was accumulated in a membrane-spanning fashion, the subsequent import of AAC and PiC was inhibited by 40 and 50%, respectively.

These observations demonstrate that accumulation of F1β in a membrane-spanning fashion efficiently blocks the import of other mitochondrial preproteins with an N-terminal matrix-targeting signal. Import of proteins, however, without matrix-targeting signals into the inner membrane, the intermembrane space, and into the outer membrane is only partly affected. Thus, functional outer membrane import sites seem to be present in excess over components required for the formation of translocation contact sites for protein import into the matrix. Outer membrane import sites that are not engaged in contact site formation are available for import of members of the mitochondrial carrier family via Tim22.

**Quantification and Stoichiometry of Protein Translocases**

**FIG. 2. Inhibition of protein import into mitochondria by accumulated translocation intermediates.** In a first stage reaction, mitochondria (30 µg of protein) were incubated with pF1β (0.1 µg) in the presence or absence of 100 µg anti-F1β-IgG. Subsequently, mitochondria were resolubilized and incubated in a second stage reaction for 15 min at 25 °C with 35S-labeled preproteins. Mitochondria were treated with 100 µg/ml PK. Samples were analyzed by SDS-PAGE and fluorography and quantified by densitometry of x-ray films. The amount of preprotein imported into mitochondria that did not receive anti-F1β-IgG in the first incubation was set 100%. CCCHL, cytochrome c heme lyase; PiC, inorganic phosphate carrier; AAC, ADP/ATP carrier; Cyt c1, cytochrome c1; Rieske FeS, Rieske iron-sulfur protein.

Tom40 comprised 0.2% of the total radioactivity incorporated into mitochondrial protein. Tim23 comprised 0.04%, and Tim22 comprised about 0.025% of the total radioactivity. Considering differences in the specific activity of these proteins due to the number of incorporated [35S]methionine and [35S]cysteine, the molar ratio of Tom40:Tim23:Tim22 is about 5:1:0.22. This corresponds to approximately 85 pmol of Tom40, 17 pmol of Tim23, and 3.7 pmol of Tim22 per mg of mitochondrial protein. The ratio of Tom40 to Tim23 was not affected when yeast cells were grown on fermentable and nonfermentable carbon sources (not shown).

**DISCUSSION**

Translocation contact sites for import of preproteins into the matrix can be saturated with chemical amounts of a preprotein, pF1β. Accumulation of this preprotein in a membrane-
spanning fashion specifically blocked subsequent import of preprotein with a matrix-targeting signal, whereas import of precursors without such a signal into the intermembrane space, and into the inner membrane was only partly blocked. This suggests that Tim23-Tim17 complexes, which mediate translocation of matrix-targeted preproteins across the inner membrane, are less abundant than Tom complexes, which constitute the general insertion pore for preproteins in the outer membrane (23). We have determined that mitochondria contain about 85 pmol of Tom40, 17 pmol of Tim23, and 3.7 pmol of Tim22 per mg of mitochondrial protein. The molar ratio of these components seems to be independent of the functional state of the mitochondria. Although the stoichiometry of the quantified components within functional Tom and Tim translocases is not known, it is likely that Tom complexes are in excess over Tim23-Tim17 complexes. The native molecular mass of the Tom complex is about 400 kDa (24, 25). Thus, the Tom complex could contain about five copies of Tom40 if the other Tom components (26) are present in only single copies. Accordingly, mitochondria should contain at least 17 pmol of Tom complexes per mg of protein. The Tim23-Tim17 complex functions as a dimeric or higher oligomeric species (27), corresponding to 8.5 pmol/mg. Tom complexes should therefore be present in at least 2-fold molar excess over Tim17-23 complexes. In agreement with this estimation, saturation of contact sites between Tom complexes and Tim17-23 complexes by matrix-targeted pFβ almost completely blocked subsequent protein import into the matrix. The remaining Tom complexes that are not engaged in formation of contact sites are obviously available for import pathways that are independent of Tim23-Tim17 complexes. These are pathways for import of proteins into the outer membrane and the intermembrane space as well as the pathway for import of proteins into the inner membrane via the Tim22 complex. Protein import via these pathways was reduced by about 40% when translocation contact sites were saturated by pFβ. This reduction could reflect an accumulation of a fraction of the precursors at receptors of Tom complexes that are blocked by membrane-spanning Fβ.

In conclusion, the interaction of the Tom machinery with translocases in the inner membrane is highly dynamic: a preprotein initially binds to a Tom complex and then recruits the appropriate machinery required for its further import and mitochondrial sorting.

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