Insertion of Mitochondrial DNA-encoded $F_1F_0$-ATPase Subunit 8 across the Mitochondrial Inner Membrane in Vitro*

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Cytochrome oxidase subunits I, II, and III, the mitochondrial DNA-encoded proteins, are inserted across the inner membrane by the Oxa1p-containing translocator in a membrane potential-dependent manner. Oxa1p is also involved in the insertion of the cytoplasmically synthesized precursor of Oxa1p itself into the inner membrane from the matrix via the conservative sorting pathway. The mechanism of insertion of the other mitochondrially synthesized proteins, however, is unexplored. The insertion of the mitochondrial DNA-encoded subunit 8 of $F_1F_0$-ATPase (Su8) across the inner membrane was analyzed in vitro using the inverted inner membrane vesicles and the Escherichia coli lysate-synthesized substrate. This assay revealed that the N-terminal segment of Su8 inserted across the membrane to the intermembrane space and assumed the correct trans-cis topology depending on the mitochondrial matrix fraction. This translocation reaction was similar to those of Sec-independent, direct insertion pathways of E. coli and chloroplast thylakoid membranes. (i) It required neither nucleotide triphosphates nor membrane potential, and hydrophobic forces drove the process. (ii) It did not require protease-sensitive membrane components facing the matrix space. (iii) It could be inserted across liposomes in the correct topology in a matrix fraction-dependent manner. Thus, a novel mechanism conserved in bacteria and chloroplasts also functions in the insertion of Su8 across the mitochondrial inner membrane.

The majority of mitochondrial inner membrane proteins are encoded by the nucleus, synthesized in the cytoplasm, and inserted into the membrane by either the stop transfer mechanism or the conservative mechanism (1–4). A subset of proteins (e.g. 8 in Saccharomyces cerevisiae and 13 in humans) are encoded by the mitochondrial genome and are, except for mitochondrial ribosomal protein Var1, assembled co- or post-translationally into the inner membrane. These membrane proteins are inserted into the inner membrane in a manner that requires complete translocation of hydrophilic negatively charged segments across the lipid bilayer to the intermembrane space. In S. cerevisiae mitochondria, translation initiation of several proteins depends on mRNA-specific activators that mediate the functional interaction between the mRNA 5′-untranslated leaders and the mitochondrial ribosomes (5, 6). Because the activator proteins are bound to the inner membrane, translation occurs on membrane-tethered ribosomes. In this way, they might promote co-translational membrane insertion, although this remains to be verified.

Mitochondria are believed to be derived from a bacterial ancestor, and the mitochondrial protein export system resembles that of bacteria, topologically and evolutionally. The Sec-dependent export machinery that is conserved in Escherichia coli and plant thylakoid, however, is not encoded in the yeast genome. These observations suggest that there is a Sec-independent export pathway in mitochondria. Recent studies indicate that Oxa1p, a nuclear-encoded protein that is conserved from prokaryotes to eukaryotes (7–9), mediates insertion across the membrane of a subset of inner membrane proteins encoded by mitochondrial and nuclear DNA (10). In yeast, depletion of Oxa1p induces a complete loss of Cox1 activity (7). Subsequently, Oxa1p was demonstrated to be involved in topogenesis of CoxII; it mediates export of the N- and C-terminal tails of CoxII to the intermembrane space (11, 12). A recent report demonstrated that it also mediates the insertion of CoxI and CoxIII into the inner membrane (13). The accumulated evidence indicates that the Oxa1p system mediates export of the negatively charged tails or loops connecting the transmembrane domains (TMDs) across the membrane to the intermembrane space in a membrane potential-dependent manner. Also, Oxa1p might be involved in the membrane assembly of $F_1F_0$-ATPase because $F_1F_0$-ATPase activity is significantly decreased in Δoxa1 yeast cells (14). Later experiments, however, demonstrated that this was caused by rapid degradation of $F_1F_0$-ATPase subunits by the AAA-protease Yme1p in Δoxa1. Furthermore, $F_1F_0$-ATPase activity and insertion of its membrane subunits were restored in Δoxa1Δyme1 cells, suggesting that Oxa1p is not essential for the export of the $F_1F_0$-ATPase membrane subunits (15).

In bacteria, most integral membrane proteins are inserted into the inner membrane using the SRP homologue Ffh, the SRP receptor FtsY, and the SecYEG trimeric complex (16, 17). The other membrane proteins such as the M13 procoat protein and the Pseudomonas P33 coat protein are inserted across the membrane in the Sec-independent pathway; the proteins are thought to insert directly into the lipid bilayer by a mechanism

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1 The abbreviations used are: Cox, cytochrome oxidase subunit; SRP, signal recognition particle; INV, inverted mitochondrial inner membrane vesicle; PMSF, phenylmethanesulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; TMDs, transmembrane domains; T7-Su8, T7-tagged Su8; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; ATPγS, adenosine 5′-O-(thiotriphosphate); GTPγS, guanosine 5′-O-(thiotriphosphate).
driven by hydrophobic forces or, in several cases, membrane potential. The M13 procoat protein is synthesized with a cleavable signal peptide and spans the membrane once with the N-terminal region translocated to the periplasm and the C-terminal tail remains exposed to the matrix space. The insertion was driven by hydrophobic forces because it is dependent on the hydrophobicity of the TMD. Furthermore, Su8 was inserted post-translationally into liposomes in the correct topology in the membrane fraction-dependent manner. These results suggest that the Sec- and Oxa1p-independent insertion pathways, which are conserved in *E. coli* and chloroplast thylakoids, also exist in mitochondria, although the involvement of proteinaceous machinery remains to be clarified.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Rat mitochondrial DNA was prepared from isolated mitochondria as described previously (30). Rat Su8 was cloned using the polymerase chain reaction with mitochondrial DNA as a template and the following oligonucleotides as the primers: upstream primer, TTOGATCCATGCCACAATGACATACATCCAGT; and downstream primer, ATATCCGATTGATGCTGGCCATGGAAGA-GCCAA. The amplified fragment was cloned directly into pGEM-T Easy (Promega). After DNA sequencing, the mitochondrial codons were mutated to corresponding universal codons using polymerase chain reaction-dependent mutagenesis. The DNA encoding Su8 by universal codons was excised with BamHI and XhoI and cloned into pET-28a(+) (Novagen) to fuse the T7 tag sequence to the N terminus. The segment coding for T7-tagged Su8 (T7-Su8) was excised with NdeI and BamHI, and ligated to pET-3a (Novagen). TM1 and TM2 mutants were generated by the method of Kunkel (31).

**Preparation of Inverted Inner Mitochondrial Vesicles**—Rat mitochondrial inner membrane vesicles were prepared as described by Jascur (32) with a slight modification. Rat liver mitochondria, prepared as described previously (33), were diluted to a concentration of 1 mg/ml with 20 mM HEPES-KOH buffer (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), placed on ice for 30 min, and sonicated with a Branson sonicator equipped with a microtip for 15 s at 15-s intervals for a total 6 min at 0 °C. The unbroken mitochondria were removed by centrifugation at 30,000 × g for 20 min at 4 °C, and the membrane vesicles were precipitated from the supernatant by centrifugation at 100,000 × g for 1 h at 4 °C. The membrane vesicles were suspended in a small volume of 10 mM HEPES-KOH buffer (pH 7.4) containing 10 mM KCl and layered in centrifuge tubes on a linear gradient of 0.85–1.6 M sucrose in 10 mM HEPES-KOH buffer (pH 7.4) containing 10 mM KCl and centrifuged at 100,000 × g for 16 h at 4 °C. After centrifugation, the solution was collected from the top of the tubes into four fractions, and each fraction was diluted with nine volumes of buffer A (33) containing 10 mM KCl and 10 mM MgCl2 and centrifuged separately at 100,000 × g for 1 h. The recovered membrane vesicles were suspended in the same buffer, and each aliquot was subjected to Western blotting with antibodies against rat Tom40 and rat Tim17 to determine which fractions contained the mitochondrial outer and inner membranes, respectively. The lower two fractions contained the inner membrane vesicles. The orientation of the inner membrane vesicles was determined as follows. Inner membrane vesicles or mitoplasts (50 μg) were treated with or without 100 μg/ml trypsin on ice for 30 min. After the addition of 500 μg/ml soybean trypsin inhibitor, the reaction mixtures were centrifuged at 100,000 × g for 15 min to recover the membranes, which were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting with antibodies against the N-terminal intermembrane space segment of rat Tim23 and full-length rat Tim44 (34). To determine the membrane topology of endogenous Su8, 50 μg of INVs or mitoplasts were treated with 100 μg/ml proteinase K at 0 °C for 30 min. After addition of 3 mM PMSF, membrane vesicles were pelleted by centrifugation at 100,000 × g for 15 min and analyzed using Western blotting with antibodies against a synthetic peptide for the matrix-exposed C-terminal segment (12 residues) of rat Su8.

**Preparation of the Mitochondrial Matrix Fraction**—The mitochondrial matrix fraction was prepared according to the method used for the system as the substrate. Su8 is a 67-residue membrane subunit of oligomycin-sensitive ATPase and is inserted into the inner membrane once with a short N-terminal region translocated to the intermembrane space, whereas the C-terminal tail remains on the matrix side (28, 29). Using this system, Su8 can be inserted post-translationally across INVs with the correct *trans-cis* topology depending on the membrane fraction but independently of ATP, membrane potential, or protein components exposed to the matrix space. The insertion was driven by hydrophobic forces because it is dependent on the hydrophobicity of the TMD. Furthermore, Su8 was inserted post-translationally into liposomes in the correct topology in the membrane fraction-dependent manner. These results suggest that the Sec- and Oxa1p-independent insertion pathways, which are conserved in *E. coli* and chloroplast thylakoids, also exist in mitochondria, although the involvement of proteinaceous machinery remains to be clarified.
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Fig. 2. Post-translational insertion of T7-Su8 across INVs and determination of its topology within the membrane. A, the amino acid sequence and hydropathy profile (Kyte and Doolittle) of rat Su8. T7 tag was fused to the N terminus of Su8 and used for the insertion assay. B, susceptibility to the sodium carbonate (pH 11.5) extraction of the proteinase K-resistant fragments of the inserted T7-Su8. S<sup>35</sup>-T7-Su8 was subjected to the insertion reaction in the presence of 1.4 mg/ml INVs and 5 mg/ml mitochondrial matrix fraction at 37 °C for 50 min in aliquots of four reaction mixtures. Three of the reaction mixtures were treated with or without proteinase K and spun in an Ultracentrifuge to isolate membrane vesicles (P), one of which was subjected to sodium carbonate extraction followed by ultracentrifugation to separate the supernatant (S) and the precipitate (P'). All fractions were trichloroacetic acid-precipitated. A remaining aliquot was treated with proteinase K in the presence of Triton X-100 and then trichloroacetic acid-precipitated. All these fractions were subjected to SDS-PAGE and subsequent fluorography analysis. C, identification of the N- and C-terminal segments within the proteinase K-protected fragments of the inserted T7-Su8 by immunoprecipitation. The insertion reaction was performed as in B, followed by immunoprecipitation. See “Experimental Procedures” for details. D, predicted topologies in INVs of the inserted T7-Su8. IP, immunoprecipitation; IMS, intermembrane space.

Preparation of the chloroplast S-30 fraction (35) except that the extraction buffer was adjusted to pH 7.4.

Synthesis of T7-Su8 and Its Insertion into INVs in Vitro—T7-Su8 was synthesized in the transcription-translation coupled E. coli S30 system according to the method of Yamane et al. (36) with a slight modification, except that 7.14 units/μl T7 RNA polymerase (Takara), 11.7 KBq/μl [35S]protein-labeling mix (PerkinElmer Life Sciences), and 0.5 μg of plasmid DNA were used. The reaction was conducted at 37 °C for 45 min, and then 2 mM puromycin was added to the mixture, and the mixture was incubated for 10 min to terminate the reaction. The insertion reaction of T7-Su8 across INVs was performed as described above, the membrane vesicles were dissolved in 100 μl of 0.1 M sodium carbonate (pH 11.5) and incubated on ice for 20 min. The mixture was centrifuged at 100,000 × g for 50 min in aliquots of four reaction mixtures. Three of the reaction mixtures were treated with or without proteinase K and spun in an Ultracentrifuge to isolate membrane vesicles (P), one of which was subjected to sodium carbonate extraction followed by ultracentrifugation to separate the supernatant (S) and the precipitate (P'). All fractions were trichloroacetic acid-precipitated. A remaining aliquot was treated with proteinase K in the presence of Triton X-100 and then trichloroacetic acid-precipitated. All these fractions were subjected to SDS-PAGE and subsequent fluorography analysis. C, identification of the N- and C-terminal segments within the proteinase K-protected fragments of the inserted T7-Su8 by immunoprecipitation. The insertion reaction was performed as in B, followed by immunoprecipitation. See “Experimental Procedures” for details. D, predicted topologies in INVs of the inserted T7-Su8. IP, immunoprecipitation; IMS, intermembrane space.

Y. Akiyama and K. Ito, personal communication.
Preparation of Liposomes—Liposomes were prepared by the method of Vergeres et al. (38) with a slight modification. A mixture of cardiolipin and phosphatidylcholine (1:4, w/w) in organic solvents was evaporated by flushing N2 and suspended with vortexing in buffer F (20 mM HEPES-KOH (pH 7.4) containing 100 mM NaCl and 1 mM EDTA) containing 170 mM sucrose. The mixture was subjected to freezing-thawing five times, diluted with five volumes of buffer F, and spun in an Ultracentrifuge at 100,000 \( g \) for 30 min at 22 °C to obtain liposomes, which were suspended in buffer F. Phospholipid concentrations were determined by measuring lipid phosphate by the method of Eibl and Lands (39). For the membrane insertion assay, liposomes containing the same amounts of lipid phosphate as that of INVs were used.

Depletion of ATP—In vitro translation products, mitochondria matrix fractions, and INVs were treated separately with 15 mM deoxyglucose and 50 units/ml hexokinase at 37 °C for 10 min prior to the insertion reaction.

RESULTS

In Vitro Assay System—To study the mechanisms of insertion of mitochondrial DNA-encoded proteins across the inner membrane, we developed an in vitro assay system. The mitochondrial DNA-encoded subunit 8 of \( F_1F_0 \)-ATPase (Su8) was used as the substrate. Su8 is a 67-residue, single-spanning membrane protein of \( F_1F_0 \)-ATPase subunits with an N-terminal 8-residue segment that is exposed to the intermembrane space and a C-terminal tail of 41 residues exposed to the matrix space (28, 29). The template DNA encoding T7-Su8 by universal codons was subjected to transcription-translation with \( E. \) coli S30 extract in the presence of \( ^{35} \)S-methionine, and the synthesized \( ^{35} \)S-T7-Su8 was subjected to the insertion reaction with INVs and the mitochondrial matrix fraction. The reaction mixtures were then treated with protease K, and the protected fragments in the INVs were analyzed using a Fluoromager to assess insertion (Fig. 1A). The orientation of the INVs was first examined using proteolysis followed by immunoblot analysis with antibodies against Tim44 and the intermembrane space segment of Tim23. Rat Tim44 localizes to the inner membrane exposing the bulk portion to the matrix side,

Fig. 3. Insertion of T7-Su8 across INVs requires mitochondrial matrix fraction. A, time course of insertion of T7-Su8 in the absence of the mitochondrial matrix fraction. The insertion reaction was performed for the indicated times in the absence of the mitochondrial matrix fraction. B, time course of insertion of T7-Su8 in the presence of the mitochondrial matrix fraction. The insertion reaction was performed for 20 min in 5 mg/ml matrix fraction. C, quantification of the band A, B, and C topologies during the insertion of T7-Su8 in the presence of the indicated concentrations of the mitochondrial matrix fraction. Bands A, B, and C in the fluorograms in panels A and B were quantified using a Bioimage Analyzer.
whereas Tim23 is a polytopic integral inner membrane protein with the N-terminal segment exposed to the intermembrane space (34). As expected, Tim44 in the mitoplasts was resistant to externally added trypsin, whereas the intermembrane segment of Tim23 was completely removed (Fig. 1B). In the INVs, in contrast, ~75% of Tim44 was sensitive, and ~90% of Tim23 was resistant to the trypsin treatment. These results indicate that ~75% or more (see Fig. 1C, for example) of INVs had an inside-out orientation. The topology of endogenous Su8 in the isolated INVs was then determined using protease susceptibility assays. The mitoplasts or INVs were treated with proteinase K, and the remaining Su8 was analyzed using SDS-PAGE followed by immunoblotting with antibodies against a synthetic peptide for the C terminus of Su8. As shown in Fig. 1C, Su8 in the mitoplasts was resistant to proteinase K digestion, whereas it was completely digested in the INVs, thus confirming the reported topography of the C terminus. Determination of the N terminus of Su8 exposed to the intermembrane space was unsuccessful, however, because the reactivity of the antibodies prepared against a synthetic peptide for the N-terminal segment was too weak to detect Su8 fragments. Exposure of the N terminus of Su8 to the intermembrane space was later verified with T7-Su8 in the in vitro assay (see below).

**In Vitro Synthesized T7-Su8 Is Inserted across INVs Post-**
To assess the insertion of Su8 across INVs, we constructed a T7-Su8 fusion protein in which the T7 tag was fused to the N terminus of Su8 (Fig. 2A). If in vitro synthesized T7-Su8 was inserted correctly across the INVs, the T7 tag should be protected from protease digestion. This was indeed the case. Proteinase K treatment of the INVs incubated with T7-Su8 in the presence of the matrix fraction produced the three protected bands A, B, and C (Fig. 2B), which were resistant to extraction with 100 mM sodium carbonate (pH 11.5). Furthermore, all three bands were digested by proteinase K in the presence of Triton X-100. These results indicate that bands A, B, and C were firmly integrated into the INVs and protected by the membrane. The same three bands were detected when in vitro synthesized T7-Su8 was incubated co-translationally with the INVs, although they significantly inhibited the transcription-translation reaction (data not shown). The topology of these bands in the INVs was then assessed using immunoprecipitation with antibodies against T7 and the C-terminal segment of Su8, which revealed that bands A and B contained the T7 tag, band A contained the C-terminal segment, but band C contained neither the T7 tag nor the C-terminal segment (Fig. 2C). Thus, the membrane topology of bands A, B, and C was deduced as shown in Fig. 2D; bands A and B were inserted across the membrane with the N-terminal portion exposed to the lumenal space (i.e. corresponding to the intermembrane space side), whereas band C was inserted into the membrane with both the N- and C-terminal domains remaining on the outer surface of the INVs (i.e. corresponding to the matrix side). It is not known why the C-terminal segment of band A was resistant to proteinase K. The positive net charges in the C-terminal domain (Fig. 2A) might interact with the negatively charged phospholipid head groups and prevent access to the protease (21). To further examine the integration process, the translocation time course was examined. As shown in Fig. 3 (B and C), the majority of T7-Su8 was initially inserted into the membrane in the band C topology, which then decreased gradually. In contrast, T7-Su8 with the A or B topologies increased over time for 50 min in a manner reciprocal to band C. The N terminus of band C seemed to translocate over time across the membrane to assume the topology of bands A and B.

**Translationally**—To assess the insertion of Su8 across INVs, we constructed a T7-Su8 fusion protein in which the T7 tag was fused to the N terminus of Su8 (Fig. 2A). If in vitro synthesized T7-Su8 was inserted correctly across the INVs, the T7 tag should be protected from protease digestion. This was indeed the case. Proteinase K treatment of the INVs incubated with T7-Su8 in the presence of the matrix fraction produced the three protected bands A, B, and C (Fig. 2B), which were resistant to extraction with 100 mM sodium carbonate (pH 11.5). Furthermore, all three bands were digested by proteinase K in the presence of Triton X-100. These results indicate that bands A, B, and C were firmly integrated into the INVs and protected by the membrane. The same three bands were detected when in vitro synthesized T7-Su8 was incubated co-translationally with the INVs, although they significantly inhibited the transcription-translation reaction (data not shown). The topology of these bands in the INVs was then assessed using immunoprecipitation with antibodies against T7 and the C-terminal segment of Su8, which revealed that bands A and B contained the T7 tag, band A contained the C-terminal segment, but band C contained neither the T7 tag nor the C-terminal segment (Fig. 2C). Thus, the membrane topology of bands A, B, and C was deduced as shown in Fig. 2D; bands A and B were inserted across the membrane with the N-terminal portion exposed to the lumenal space (i.e. corresponding to the intermembrane space side), whereas band C was inserted into the membrane with both the N- and C-terminal domains remaining on the outer surface of the INVs (i.e. corresponding to the matrix side). It is not known why the C-terminal segment of band A was resistant to proteinase K. The positive net charges in the C-terminal domain (Fig. 2A) might interact with the negatively charged phospholipid head groups and prevent access to the protease (21). To further examine the integration process, the translocation time course was examined. As shown in Fig. 3 (B and C), the majority of T7-Su8 was initially inserted into the membrane in the band C topology, which then decreased gradually. In contrast, T7-Su8 with the A or B topologies increased over time for 50 min in a manner reciprocal to band C. The N terminus of band C seemed to translocate over time across the membrane to assume the topology of bands A and B.

**Insertion of T7-Su8 across the Inner Membrane Requires the Mitochondrial Matrix Fraction**—Post-translational membrane integration of T7-Su8 in the correct topology strictly depended.
on the mitochondrial matrix fraction (Figs. 3, A and C, and 4). In the absence or the presence of the heat-treated matrix fraction, only a trace amount of bands A and B was detectable (Fig. 4A). In contrast, only a slight retardation in the accumulation of the C topology was observed in the absence of the matrix fraction (Fig. 3, A and C). The accumulation of T7-Su8 with the A and B topologies was strictly dependent on the amount of the matrix fraction added to the reaction (Fig. 4C). These results clearly indicate that the matrix fraction was required for the insertion of the N terminus of T7-Su8 across the membrane.

We then examined whether T7-Su8 with the band C topology could be chased to the band A and B topologies in a matrix fraction-dependent manner. The INVs that were incubated with T7-Su8 in the absence of the matrix fraction were recovered by centrifugation and incubated further with or without the matrix fraction, followed by proteinase K digestion (Fig. 5A). As shown in Fig. 5B (B and C), in the absence of the matrix fraction, a small amount of band C was converted to bands A and B, the background activity of which was probably derived from contamination by the E. coli S-30 fraction. The mitochondrial matrix fraction significantly stimulated the conversion (Fig. 5B, B and C), thus indicating that membrane translocation of the N-terminal segment of T7-Su8 strictly depended on the matrix fraction.

Insertion of T7-Su8 across the Membrane Depends on the Hydrophobicity of the TMD—Because hydrophobic forces have a crucial role in membrane translocation of the E. coli Sec-independent pathway (17, 18, 24, 26), we examined the role of hydrophobic forces on the insertion of T7-Su8 across INVs. We constructed the T7-Su8 mutants TM1 and TM2, in which hydrophobic residues in the former (TM1) and the latter (TM2) half of the TMD were replaced by hydrophilic residues (Fig. 6A). Membrane integration activity greatly deteriorated in TM1 and was completely lost in TM2 (Fig. 6B), indicating that hydrophobicity of the TMD is crucial for the matrix fraction-dependent membrane insertion of T7-Su8.

ATP or Membrane Potential Is Not Required for Insertion of T7-Su8 across the Inner Membrane—Oxa1p mediates export of the negatively charged N- or C-terminal tails or the loops connecting the TMDs to the intermembrane space in CoxII and nuclear-encoded preOxa1p (10, 11). The N terminus of Pf3 coat protein is exported in a membrane potential-dependent manner (25). Therefore, we examined whether the export of the N terminus of T7-Su8 depends on the membrane potential and/or ATP. The insertion reaction was temperature-dependent (Fig. 7A), and
neither ATP depletion from the reaction mixture nor dissipation of the membrane potential with ionophores inhibited the export (Fig. 7, B and C). Furthermore, neither ATPγS nor GTPγS affected the export efficiency (data not shown). These and the above results indicate that neither the membrane potential nor a nucleotide triphosphate-coupled system was involved in the translocation of the N terminus of T7-Su8 across the inner membrane and suggest that this process was driven mainly by hydrophobic forces. The N terminus of T7-Su8 translocated with a slightly faster time course when succinate was present in the assay system, suggesting that the membrane potential slightly affects the translocation, although it is not essential for the translocation (data not shown).

**Integration of T7-Su8 into Trypsin-treated INVs**—We examined whether protein components exposed to the outer surface of INVs are involved in the translocation of the N terminus of T7-Su8. Trypsin treatment of the vesicles did not inhibit the matrix fraction-assisted translocation (Fig. 7D), indicating that the membrane components exposed to the matrix surface are not involved in the translocation process. The possibility that the components or the segments deeply embedded in the inner membrane take part in this process, however, cannot be ruled out.

**T7-Su8 Can Be Integrated into Liposomes in a Matrix Fraction-dependent Manner**—Several proteins in the Sec-independent pathway of *E. coli* (e.g., Pf3 coat protein) and chloroplast thylakoids (e.g., CF0II, PsbX, and PsbW; see “Discussion”) insert directly across the lipid bilayer (17). We therefore examined whether T7-Su8 can translocate across synthetic liposomes. As shown in Fig. 8, T7-Su8 was inserted across liposomes over time in the same topologies as in the case for INVs (Fig. 8A), and this reaction was dependent on the matrix fraction (Fig. 8, B and C), although the kinetics of the insertion were slightly different between them (compare Fig. 3C with Fig. 8C). The precusor-product relation between band C and bands A plus B was not apparent in liposomes, suggesting that the translocation step might be rate-limiting in the absence of the membrane proteins. In any event, these results indicate that T7-Su8 can be inserted directly across the lipid bilayer in a matrix fraction-dependent manner.

**DISCUSSION**

The mitochondrialy synthesized proteins CoxI, CoxII, and CoxIII and the nuclear encoded mitochondrial precursor protein with a bipartite sorting signal, preOxa1p, are inserted across the membrane by Oxa1-dependent translocation in a membrane potential-dependent manner (10, 11, 13). Nevertheless, the insertion mechanism of the other mitochondrialy synthesized proteins is not well understood.

We developed an in vitro insertion assay system and analyzed the insertion of Su8 across the INVs. This assay system revealed that T7-Su8 is first inserted into the outside of INVs in a cis-cis orientation, and then the N terminus translocates in a matrix fraction-dependent manner to the luminal side to assume the correct trans-cis orientation. This insertion can proceed post-translationally and neither requires ATP nor membrane potential, although the membrane potential slightly stimulates the reaction. Because the insertion activity is significantly impaired by decreasing hydrophobicity of the TMD, hydrophobic forces seem to have a crucial role in membrane translocation, whereas electrical force has an auxiliary function to translocate the negatively charged N-terminal segment. Furthermore, trypsin treatment of INVs does not interfere with the translocation activity, suggesting that surface (i.e. matrix side) exposed protein regions of the translocation components, if any, are not essential. Because Oxa1p exposes protease-susceptible charged segments to the outer surface of INVs, Oxa1 is probably not involved in the insertion of T7-Su8.

In this respect, the insertion of T7-Su8 across membranes resembles that of the Pf3 coat protein of *E. coli* inner membrane and ATPase subunit CF0II and PsbX and PsbW of photosystem II of *Arabidopsis* thylakoids. CF0II, PsbX, and PsbW are synthesized in the cytosol with a bipartite targeting signal, and after processing of the stroma-targeting signal they are inserted from the stroma across the membrane, independent of SRP, SecA, SecYE, nucleotide triphosphates, and membrane potential (16, 17, 40–42). Protease treatment of thylakoid membrane does not impair their insertion across the membranes (41). Of note, Pf3 coat protein insertion strictly depends on membrane potential. This is probably because the hydrophobicity of the TMD is insufficient to drive insertion, because an extended TMD allows for membrane insertion in the absence of membrane potential (26). Whether the insertion of these proteins across the membranes proceeds in the membrane translocator-mediated pathway or in the translocator-independent direct insertion mechanism still remains to be clarified. In any event, our present results suggest that a unique membrane translocation mechanism that is conserved in *E. coli* and chloroplasts is also present in mitochondria.

The function of the mitochondrial matrix fraction remains to be established. Translocation of the N terminus of T7-Su8 depends strictly on the mitochondrial matrix fraction, whose activity is inactivated by heat treatment. We speculate that the matrix fraction makes the conformation of T7-Su8 translocation-competent. Because the initial membrane insertion of T7-Su8 in the cis-cis orientation (band C orientation) does not require the matrix fraction, newly synthesized T7-Su8 should assume the insertion-competent conformation. Rather, the matrix fraction is required for the subsequent step of translocation of the T7-Su8 N terminus across the membrane. Another possibility might be that the matrix fraction functions similarly to Sec A and drives the insertion of the substrate protein into the lipid bilayer. This is not consistent with the present results, however, because T7-Su8 was inserted across the membrane spontaneously in the presence of the matrix fraction. Additional analysis is required.

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**REFERENCES**

1. Stuart, R. A., and Neupert, W. (1996) *Trends Biochem. Sci.* 21, 261–267.
2. Voos, W., Martin, H., Krimmer, T., and Pfanner, N. (1999) *Biochem. Biophys. Res. Commun.* 263, 77–81.
3. Rassow, J., Dekker, P. J., van Wilpe, S., Meijer, M., and Soli, J. (1999) *J. Mol. Biol.* 290, 105–120.
4. Tokatlidis, K., and Schatz, G. (1999) *J. Biol. Chem.* 274, 35285–35288.
5. Sanchirico, M. E., Fox, T. D., and Mason, T. L. (1998) *EMBO J.* 17, 5796–5804.
6. Michaelis, U., Korte, A., and Rodel, G. (1991) *Mol. Gen. Genet.* 230, 177–185.
7. Bonnefoy, N., Chalvet, F., Hamel, P., Sliminski, P. P., and Dujardin, G. (1994) *J. Mol. Biol.* 239, 201–212.
8. Boysen, N., Kermognant, M., Groudinsky, O., Minet, M., Sliminski, P. P., and Dujardin, G. (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91, 11978–11982.
9. Sundberg, E., Slagter, J. G., Fridborg, I., Cleary, S. P., Robinson, C., and Coupland, G. (1997) *Plant Cell* 9, 717–730.
10. Hell, K., Herrmann, J. M., Pratje, E., Neupert, W., and Stuart, R. A. (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95, 2250–2255.
11. He, S., and Fox, T. D. (1997) *Mol. Biol. Cell* 8, 1449–1460.
12. Hell, K., Herrmann, J. M., Pratje, E., Neupert, W., and Stuart, R. A. (1997) *FEBS Lett.* 418, 367–370.
13. Hell, K., Neupert, W., and Stuart, R. A. (2001) *EMBO J.* 20, 1281–1288.
14. Altamura, N., Capitanio, N., Bonnefoy, N., Papa, S., and Dujardin, G. (1996) *FEBS Lett.* 382, 111–115.
15. Lemaire, C., Hamel, P., Velours, J., and Dujardin, G. (2000) *J. Biol. Chem.* 275, 23471–23475.
16. Dalbey, R. E., and Robinson, C. (1999) *Trends Biochem. Sci.* 24, 17–22.
17. Dalbey, R. E., and Rahn, A. (2000) *Annu. Rev. Cell Dev. Biol.* 16, 51–87.
18. Kuhr, A., Krell, G., and Wickner, W. (1986) *EMBO J.* 5, 3681–3685.
19. Kuhr, A. (1987) *Science* 238, 1413–1415.
20. Date, T., Goodman, J. M., and Wickner, W. T. (1980) *Proc. Natl. Acad. Sci. U.S.A.* 77, 4669–4673.
21. Gallusser, A., and Kuhn, A. (1999) *EMBO J.* 9, 2723–2729.
22. Geller, B. L., and Wickner, W. (1985) *J. Biol. Chem.* 260, 13281–13285.
23. Wolfe, P. B., Rice, M., and Wickner, W. (1985) *J. Biol. Chem.* 260, 1836–1841.
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24. Cao, G., Kuhn, A., and Dalbey, R. E. (1995) *EMBO J.* **14**, 866–875
25. Kiefer, D., Hu, X., Dalbey, R., and Kuhn, A. (1997) *EMBO J.* **16**, 2197–2204
26. Kiefer, D., and Kuhn, A. (1999) *EMBO J.* **18**, 6299–6306
27. Samuelson, J. C., Chen, M., Jiang, F., Moller, I., Wiedmann, M., Kuhn, A., Phillips, G. J., and Dalbey, R. E. (2000) *Nature* **406**, 637–641
28. Hekman, C., Tomich, J. M., and Hatefi, Y. (1991) *J. Biol. Chem.* **266**, 13564–13571
29. Oda, T., Futaki, S., Kitagawa, K., Yeshihara, Y., Tani, I., and Higuti, T. (1989) *Biochem. Biophys. Res. Commun.* **165**, 449–456
30. Welker, C., Dooley, S., and Blin, N. (1989) *Gene (Amst.)* **83**, 169–172
31. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 488–492
32. Jascur, T. (1991) *Methods Cell Biol.* **34**, 359–368
33. Hashiya, N., Alam, R., Sakasegawa, Y., Sakaguchi, M., Mihara, K., and Omura, T. (1993) *EMBO J.* **12**, 1579–1586
34. Ishihara, N., and Mihara, K. (1998) *Biochem. (Tokyo)* **123**, 722–732
35. Hirose, T., and Sugiu, M. (1996) *EMBO J.* **15**, 1687–1695
36. Yamane, K., Ichihara, S., and Mizushima, S. (1987) *J. Biol. Chem.* **262**, 2358–2362
37. Schagger, H., and von Jagow, G. (1987) *Anal. Biochem.* **166**, 368–379
38. Vergeres, G., Munenti, S., Weber, T., and Sturzinger, C. (1995) *J. Biol. Chem.* **270**, 19879–19887
39. Eibl, H., and Lands, W. E. (1969) *Anal. Biochem.* **30**, 51–57
40. Michl, D., Robinson, C., Shackleton, J. B., Herrmann, R. G., and Klosgen, R. B. (1994) *EMBO J.* **13**, 1310–1317
41. Kim, S. J., Robinson, C., and Mant, A. (1998) *FEBS Lett.* **424**, 105–108
42. Kim, S. J., Jansson, S., Hoffman, N. E., Robinson, C., and Mant, A. (1999) *J. Biol. Chem.* **274**, 4715–4721
43. Kyte, J., and Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132