Interaction of Tim23 with Tim50 Is Essential for Protein Translocation by the Mitochondrial TIM23 Complex

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The TIM23 complex is the major translocase of the mitochondrial inner membrane responsible for the import of essentially all matrix proteins and a number of inner membrane proteins. Tim23 and Tim50, two essential proteins of the complex, expose conserved domains into the intermembrane space that interact with each other. Here, we describe in vitro reconstitution of this interaction using recombinantly expressed and purified intermembrane space domains of Tim50 and Tim23. We established two independent methods, chemical cross-linking and surface plasmon resonance, to track their interaction. In addition, we identified mutations in Tim23 that abolish its interaction with Tim50 in vitro. These mutations also destabilized the interaction between the two proteins in vivo, leading to defective import of preproteins via the TIM23 complex and to cell death at higher temperatures. This is the first study to describe the reconstitution of the Tim50-Tim23 interaction in vitro and to identify specific residues of Tim23 that are vital for the interaction with Tim50.

Maintenance and growth of mitochondria depend on the constitutive import of newly synthesized proteins that are encoded by nuclear DNA, translated on cytoplasmic ribosomes as precursors and eventually taken up from the cytosol. The latter process is mediated by several complex sorting and import machineries, protein translocases, and insertases that are located in the outer and inner mitochondrial membranes (1–3). The port of entry for almost all of the precursor proteins is the TOM (translocase of the outer mitochondrial membrane) complex. Once the precursor protein has started to cross the outer membrane through the TOM complex, sorting signals trigger transport to its final destination, a process facilitated by one of the various import complexes (2, 4–6).

The TIM23 (translocase of the inner mitochondrial membrane) complex handles the import of precursor proteins that contain N-terminal targeting signals, which include proteins destined for the matrix as well as some of the inner membrane and intermembrane space (IMS)3 proteins. The TIM23 complex can be distinguished into two functionally defined parts: a core complex comprised of integral membrane proteins whose subunits are firmly associated with the membrane and the import motor. The core complex comprises three essential proteins: Tim17, Tim23, and Tim50, which make up the receptors and the translocation channel of the complex. Initial translocation of the N-terminal presequence across the inner membrane requires the membrane potential ΔΨ. Completion of translocation across the inner membrane, however, requires the action of the import motor (2, 7). The import motor consists of an ATP-hydrolyzing chaperone, the 70-kDa mitochondrial heat shock protein (mtHsp70), and Tim44, which recruits the latter to the TIM23 complex (8–10). Other key players in the import motor, the co-chaperones Tim14/Pam18–Tim16/Pam16 (11–15), and the nucleotide exchange factor Mge1 (16–18) modulate the activity of mtHsp70 and thereby regulate the binding of the chaperone to the incoming unfolded precursor proteins. Finally, two nonessential membrane proteins, Pam17 and Tim21, modulate the activity of the TIM23 complex in an antagonistic manner. How exactly these proteins exert their function, however, is currently controversial (19, 20).

Tim50 is anchored in the inner membrane with one transmembrane helix and comprises a large C-terminal hydrophilic domain (∼350 amino acid residues) that is exposed to the IMS. Presumably this domain is the first component to interact with the precursor protein emerging from the TOM complex and to direct it to the TIM23 import channel (21–23). Tim23 consists of an N-terminal region of ∼100 amino acid residues that is hydrophilic and exposed to the IMS and of a C-terminal hydrophobic domain that spans the inner membrane with four transmembrane helices comprising ∼120 residues (2, 4, 6). The first ∼20 residues of Tim23 are found on the mitochondrial surface (20, 22, 28). Tim23 and Tim50 were proposed to interact via their soluble IMS domains (21, 22). Using in vivo chemical cross-linking, two regions on Tim23 were recently identified that mediate interaction with Tim50. The first one lies in the N-terminal domain, and the second one lies in the first transmembrane helix (24).

3 The abbreviations used are: IMS, intermembrane space; DSS, disuccinimidyl suberate; TEV, tobacco etch virus; mtHsp70, 70-kDa mitochondrial heat shock protein; PK, proteinase K; CBB, Coomassie Brilliant Blue.

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**The Tim23-Tim50 Interaction**

We set out to reconstitute the interaction between Tim23 and Tim50 in vitro. We prepared recombinant purified intermembrane space domains of two proteins and studied their interaction by cross-linking and surface plasmon resonance measurements. Also, we identified mutations in Tim23 that abolished its interaction with Tim50. Mitochondria harboring Tim23 with these mutations were deprived in their ability to import precursor proteins via the TIM23 complex. This is the first study to reconstitute the Tim23-Tim50 interaction in vitro from recombinant proteins and to identify specific residues on Tim23 that are essential for it.

**EXPERIMENTAL PROCEDURES**

**Materials**—Proteinase K (P-6556), V₈ proteinase (P-2922), and DNase (D-5025) were purchased from Sigma. Disuccinimidyld substrate (DSS; 21555) was from Pierce, and complete EDTA-free protease inhibitor mixture (13625900) was from Roche Applied Science.

**Construction of NusA-Tim23IMS and NusA-Tim50IMS**—The sequences encoding the soluble domains of yeast Tim23 (amino acids 1-96) and Tim50 (amino acids 133-476) were amplified by PCR, with a Saccharomyces cerevisiae genomic DNA as a template and the primers listed in Table 1. The PCR products were cloned into a modified pET-43.1a(+) vector containing octahistidine-tagged NusA protein (25) (kindly provided by Dr. Joel Hirsch) and a cleavage site for the TEV protease. The construct was engineered in such a way that the histidine tag and NusA were removed upon TEV protease treatment. The *Escherichia coli* strain BL21(DE3) was used as a host for the expression of NusA-Tim23IMS or NusA-Tim50IMS fusion proteins.

**Purification of Tim23IMS and Tim50IMS**—Bacteria were grown for ~3 h at 37 °C in 6 L of LB medium containing 100 μg/ml ampicillin. Upon reaching an A₆₀₀ of 0.6, the temperature was lowered to 16 °C, and protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside overnight. Bacteria were collected by centrifugation at 5,000 rpm for 10 min and homogenized in 60 ml of lysis buffer containing 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1% Triton X-100, 20 mM imidazole, 0.05 mg/ml lysozyme, 45 mg/ml DNase, 2 mM phenylmethylsulfonyl fluoride, and one tablet of complete EDTA-free protease inhibitor mixture. After incubation for 15 min on ice, the bacteria were disrupted using a microfluidizer. Insoluble material was removed by centrifugation at 14,000 rpm for 30 min at 4 °C. The soluble fraction was loaded onto a nickel-nitritotriacetic acid-agarose column (20 ml) pre-equilibrated with buffer A (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 20 mM imidazole). The column was washed with three volumes of buffer A until a stable base line was reached. The fractions were eluted with a linear gradient (0–50%, 90 ml) of buffer B (buffer A containing 1 M imidazole). The fractions enriched with the protein were pooled and treated with TEV protease (TEV-protein, 1:40 w/w) overnight at 4 °C in a dialysis bag against 5 liters of buffer A. The protein was then passed over a second nickel-nitritotriacetic acid-agarose column to remove in one step the TEV protease, which contained an uncleavable octahistidine tag, NusA, which remained after digestion of the fusion protein and also contained the histidine tag, along with the nondigested material and nonspecifically bound proteins. Fractions enriched with the desired protein were concentrated and loaded onto a HiLoad 16/60 Superdex 200 column (total bead volume, 90 ml; Amersham Biosciences) pre-equilibrated with 20 mM NaHEPES, pH 7.4, 200 mM NaCl, and 5% glycerol at a flow rate of 1 ml/min. Fractions with the desired protein were concentrated using Vivaspin protein concentrators (Vivascience; molecular weight cut-off of 5,000 for Tim23IMS and 10,000 for Tim50IMS), divided into aliquots, and flash-frozen in liquid N₂. All of the purification procedures were carried out at 4 °C.

**Mutagenesis**—Site-directed mutants were created according to the protocol of Stratagene, using the primers listed in Table 1.

**Circular Dichroism**—All of the CD measurements were carried out with an Aviv CD spectrometer model 202 over the range of 260-190 nm at a scan rate of 1 nm/s, using a cell with a path length of 0.1 mm. Each spectrum is an average of five scans. The raw data were corrected by subtracting the contribution of the buffer to the CD signal. The data were smoothed and converted to molar ellipticity units. The measurements were taken at a constant temperature of 25 °C in buffer containing 20 mM NaHEPES, pH 7.4, 200 mM NaCl, and 5% glycerol, with a protein concentration of 1 mg/ml.

**Thermal Aggregation Assay for Tim23IMS and Tim50IMS**—Aliquots (0.4 mg/ml) of Tim23IMS or Tim50IMS in 20 mM NaHEPES, pH 7.4, 200 mM NaCl, 5% glycerol were incubated at different temperatures for 15 min and then centrifuged at 14,000 rpm for 5 min at room temperature to obtain aggregated (pellet) and soluble (supernatant) fractions. The proteins in each fraction were analyzed by SDS-PAGE and staining with Coomassie Brilliant Blue (CBB).

**Limited Proteolysis of Tim23IMS and Tim50IMS**—V₈ Protease (0.01 mg/ml) was used to digest 0.2 mg/ml Tim23IMS or Tim50IMS. PK (0.2 μg/ml) was added to 0.4 mg/ml protein. The reactions were allowed to proceed on ice in 20 mM NaHEPES, pH 7.4, and 200 mM NaCl. Time-dependent proteolysis of proteins was followed by SDS-PAGE and CBB staining.

**Cross-linking Experiments**—Cross-linking reactions were carried out with 1 mM DSS at room temperature for 1 h in 20 mM NaHEPES, pH 7.4, 200 mM NaCl, 5 mM MgCl₂, 50 mM KCl, and 5% glycerol at a protein concentration of 10 μM. The reaction was stopped by the addition of SDS-containing sample buffer. The cross-linked products were separated by SDS-PAGE using a gradient of 8–16% acrylamide and stained with CBB.

**Surface Plasmon Resonance**—The experiments were performed with a ProteOn XPR36 instrument (Bio-Rad). This system uses an SPR-based detector to monitor formation of complexes between analytes and ligand molecules. The sensor chip (GLC) was activated for 80 s with a mixture of 0.1 M EDC and 0.025 M sulfo-NHS (TS-Pierce). Immediately after activation, 20 μg of Tim23IMS (wild type or mutant) in 1 volume of buffer C (20 mM NaHEPES, pH 7.4, 200 mM NaCl, 5% glycerol, 0.001% Tween) were diluted with 4 volumes of buffer D (10 mM sodium acetate, pH 3.5) and injected across channel/s (400 s at a flow rate of 30 μl/min). One channel, which was treated with the buffer without protein, served as a reference. Finally, the channels were blocked with 1 M ethanolamine-HCl, pH 8.5, for 200 s. Tim23IMS was coupled at response levels of ~800 RU in a chan-
nel. The chip was rotated at 90°, the channels were washed for 30 min with buffer C, and six different concentrations of Tim50 IMS were simultaneously injected at a flow rate of 30 μl/min for an association phase of 200 s, which was followed by a 600-s dissociation phase.

Yeast Strains and Media—Wild type yeast strain YPH499 was used (26). To generate the strain containing a chromosomal deletion of TIM23 rescued with Tim23 on URA plasmid, YPH499 cells were transformed with pVT-U plasmid (27) carrying the wild type copy of Tim23 under the control of the ADH promoter. The chromosomal copy of TIM23 was subsequently deleted by the KAN cassette using homologous recombination. This strain was designated Δ23 + 23URA. To generate yeast strains carrying mutant versions of Tim23 instead of wild type, Tim23 mutants were cloned into centromeric plasmid pRS315 (26) under control of the endogenous promoter and 3′ untranslated region and transformed into Δ23 + 23URA. An empty pRS315 and the plasmid carrying the wild type copy of Tim23 were transformed as controls. Yeast cells that had lost the URA plasmid were selected on medium containing 5-ﬂuoroorotic acid.

Yeast cells were normally grown on lactate medium containing 0.1% (w/v) glucose at 30 °C. To exclude possible secondary effects, for import assays yeast cells were grown at 24 °C. For drop dilution assays, 10-fold serial dilutions of yeast cells were spotted on YPG plates and incubated at 24, 30, and 37 °C for 2 days.

Miscellaneous—Coimmunoprecipitation experiments were performed according to published procedures (11). Import into isolated mitochondria was done as described previously (11) with the modification that mitochondria were preincubated for 10 min at 37 °C before the addition of radiolabeled precursor proteins.

RESULTS

Properties of Purified Tim23 IMS and Tim50 IMS—We purified the soluble intermembrane space domains of Tim23 and Tim50 after expression in E. coli. (Fig. 1A). Their secondary structure was determined by CD spectroscopy. The spectrum of Tim50 IMS showed that it was folded with a high content of α-helices and β-sheets (Fig. 1B). Tim23 IMS yielded a spectrum indicative of an unstructured protein; however, there were also indications of the presence of some structured elements (Fig. 1C). Protease digestion experiments (supplemental Fig. S1) confirmed that Tim50 IMS is a tightly folded protein, whereas Tim23 IMS is largely
unstructured. Interestingly, programs that predict secondary
structures on the basis of primary sequences suggest ~85% of
the IMS domain of Tim23 to be unstructured (supplemental
Fig. S2).

Next we examined the stability of the purified IMS domains.
The thermal stability of both proteins was assessed by following
their denaturation. Aggregation of Tim50IMS started at 50 °C
and was complete at 60 °C (Fig. 1D). Thermal denaturation was
also followed by recording the CD spectrum at 222 nm. A $T_m$
value of 52.5 °C was measured (supplemental Fig. S3). Interest-
ingly, Tim23IMS did not aggregate but remained completely
soluble at all temperatures examined (up to 80 °C) (Fig. 1E). The

FIGURE 3. Complex formation of the isolated intermembrane space domains of Tim50 and Tim23 measured by surface plasmon resonance. A, Tim23IMS
was immobilized at a density of ~800 RU in the channel. The chip was rotated at 90°, and six different concentrations of Tim50IMS analyte were injected
simultaneously at a flow rate of 30 µl/min for a 200-s association phase, followed by a 600-s dissociation phase. B, $K_D$ of the interaction, calculated by
Bia-Evaluation (BIACORE).

FIGURE 4. Interaction of Tim23IMS mutants with Tim50RMS determined by cross-linking and surface plasmon resonance. A and B, mutants Y70A and L71A,
respectively, of Tim23IMS were subjected to chemical cross-linking, as described for Fig. 2. C and D, surface plasmon resonance analysis of interaction of mutants
Y70A and L71A, respectively, of Tim23IMS with Tim50RMS. The experimental conditions were as in Fig. 3. WT, wild type.
CD spectrum of Tim23<sub>IMS</sub> remained unchanged even at temperatures as high as 80 °C (not shown). In summary, the isolated Tim50<sub>IMS</sub> protein was folded with a well defined secondary structure. In contrast, Tim23<sub>IMS</sub> appeared to be essentially unstructured.

**Reconstitution of Tim23-Tim50 Interaction Using Purified Tim23<sub>IMS</sub> and Tim50<sub>IMS</sub>**—To detect an interaction between the isolated Tim23<sub>IMS</sub> and Tim50<sub>IMS</sub> domains, we used two methods. First, they were incubated, separately or together, with the cross-linking agent DSS. The cross-linked adducts were then analyzed by SDS-PAGE. With Tim23<sub>IMS</sub> no cross-linked species were observed (Fig. 2). In contrast, Tim50<sub>IMS</sub>, after exposure to DSS, yielded a pattern comprising not only monomers but also dimers and higher oligomers (Fig. 2). Incubation of Tim23<sub>IMS</sub> in the presence of DSS and increasing concentrations of Tim50<sub>IMS</sub> led to the formation of a cross-linked product, which corresponded in size to an adduct of the two proteins. This was confirmed by Western blotting using antibodies raised against both proteins (not shown). The same result was obtained when the concentration of Tim50<sub>IMS</sub> was kept constant (10<sup>−9</sup> M), and the concentration of Tim23<sub>IMS</sub> was increased (Fig. 2B).

Concentration-dependent complex formation between Tim23<sub>IMS</sub> and Tim50<sub>IMS</sub> was also observed using surface plasmon resonance (Fig. 3A). Dissociation of the complex occurred upon washing with buffer. The <i>K</i><sub>D</i> of the interaction of Tim23<sub>IMS</sub> and Tim50<sub>IMS</sub>, calculated from the maximal SPR signal as a function of the concentrations, was 60 µM (Fig. 3B). In summary, interaction of the purified IMS domains of Tim23 and Tim50 can be demonstrated in vitro using both chemical cross-linking and SPR.

**Identification of Tim23 Mutants Whose Interaction with Tim50 Is Impaired**—To identify amino acid residues that participate in complex formation of Tim50 and Tim23, we

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**TABLE 1**

| Primers used in this study | Primers (5′-3′) |
|---------------------------|----------------|
| Tim50<sub>IMS</sub>       | GATACATCAAGCCGCAATGAGCTACTC |
| Tim23<sub>IMS</sub>       | GATACATCAAGCCGCAATGAGCTACTC |
| (wild type)               | GATACATCAAGCCGCAATGAGCTACTC |
| Tim23<sub>IMS</sub> (P60A)| GATACATCAAGCCGCAATGAGCTACTC |
| Tim23<sub>IMS</sub> (D65A)| GATACATCAAGCCGCAATGAGCTACTC |
| Tim23<sub>IMS</sub> (K66A)| GATACATCAAGCCGCAATGAGCTACTC |
| Tim23<sub>IMS</sub> (R70A)| GATACATCAAGCCGCAATGAGCTACTC |
| Tim23<sub>IMS</sub> (Y71A)| GATACATCAAGCCGCAATGAGCTACTC |
| Tim23<sub>IMS</sub> (G83A)| GATACATCAAGCCGCAATGAGCTACTC |
| Tim23<sub>IMS</sub> (D95A)| GATACATCAAGCCGCAATGAGCTACTC |
| Tim23<sub>IMS</sub> (Y70L71AA)| GATACATCAAGCCGCAATGAGCTACTC |

**TABLE 2**

Dissociation constants obtained using SPR

| Tim23 strain | <i>K</i><sub>D</i> (nM) |
|--------------|-----------------|
| Wild type    | 57.9            |
| P60A         | 156             |
| Y70A         | ND              |
| L71A         | ND              |
| G83A         | 30.6            |

*ND, no binding detected.

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**FIGURE 5**. Phenotypes of mutations in the intermembrane space domain of Tim23. A, 10-fold serial dilutions of yeast cells carrying wild type (WT) Tim23 or Y70A, L71A, or Y70L71AA mutants were spotted onto YPG plates and grown at the indicated temperatures for 2 days. B, mitochondria isolated from wild type or cells carrying the Y70L71AA double mutation in Tim23 were solubilized with digitonin and subjected to immunoprecipitation using affinity purified antibodies to Tim23 and Tim50. Antibodies from preimmune serum were used as a control. Total and immunoprecipitated fractions were analyzed by SDS-PAGE followed by immunodecoration with the indicated antibodies. C, mitochondria as above were incubated in the presence or absence of PK for the indicated times and subsequently analyzed by SDS-PAGE followed by immunodecoration with the depicted antibodies. Tom70, Tim50, and Hep1 were used as marker proteins for the outer membrane, intermembrane space, and the matrix, respectively.
The Tim23-Tim50 Interaction

FIGURE 6. Import of precursor proteins into isolated mitochondria from wild type and Tim23Y70A,L71A mutant cells. Various mitochondrial preproteins (A–E) were synthesized in vitro in the presence of [35S]methionine and incubated with isolated mitochondria. At the indicated time points the aliquots were removed and diluted into ice-cold buffer containing valinomycin to stop further import. One half of each sample was treated with protease K to digest nonimported material. The samples were subsequently analyzed by SDS-PAGE and autoradiography. A, F1β, precursor of the β-subunit of the F1 part of F0–F1-ATPase. B, b2(1–167)Δ190DHFR, precursor protein consisting of the first 167 residues of yeast cytochrome b2 with the deleted sorting signal fused to full-length dihydrofolate reductase from mouse. C, DLD(1–72)DHFR, precursor protein consisting of the first 72 residues of the yeast α-lactate dehydrogenase fused to the full-length mouse DHFR. D, precursor of subunit 5a of the cytochrome c oxidase complex from yeast. E, AAC, precursor of the ATP/ADP carrier. F, membrane potential of wild type (WT) and mutant mitochondria was assessed by fluorescence quenching with DiSC3(5).

mutated eight amino acids of Tim23IMSm which, in view of their high evolutionary conservation, were suspected to be of functional importance (supplemental Fig. S4). The Tim23IMSm domains of these mutants were prepared as described above for wild type. Upon chemical cross-linking only two mutants (Y70A and L71A) showed impaired interaction with Tim50IMSm as compared with wild type (Fig. 4, A and B, and data not shown). Analysis by SPR demonstrated virtually complete lack of interaction of Tim23IMSmY70A and Tim23IMSmL71A with Tim50IMSm (Fig. 4, C and D). The mutant P60A showed a reduction of its $K_D$ relative by a factor of three as compared with wild type (Table 2).

Examination of Tim23 Mutants in Vivo—In view of the striking loss of interaction displayed by the Y70A and L71A mutants, we analyzed them further with regard to their effects in intact yeast cells. We introduced plasmids carrying Tim23Y70A, Tim23L71A, or the double mutation into a yeast strain containing a chromosomal deletion of TIM23, which could be rescued by the wild type copy of Tim23 on the URA plasmid. After shuffling the URA plasmids out on medium containing 5-fluoro-orotic acid, yeast strains harboring only the mutant forms of Tim23 were obtained. Whereas the single mutants displayed no obvious growth defect under the conditions analyzed, the Tim23Y70A,L71A double mutant showed temperature-sensitive growth and was inviable at 37 °C (Fig. 5A). We therefore chose to further investigate only the double mutant. In mitochondria isolated from this strain grown at permissive temperature, the stability of Tim23 was unaffected, and the endogenous levels were observed to be unchanged (data not shown). The interaction between Tim23 and Tim50 in wild type and mutant mitochondria was analyzed by coimmunoprecipitation. The complex between Tim23 and Tim50 observed in wild type was not detected in the Tim23 double mutant (Fig. 5B). The same result was obtained irrespective of whether antibodies to Tim23 or to Tim50 were used for immunoprecipitation. Interestingly, the interaction of Tim23 with other subunits of the TIM23 complex such as Tim17, Tim44, and Pam17 was not affected by these mutations. This demonstrates the specific involvement of the respective residues in the interaction of the IMS domain of Tim23 with Tim50.

The first ~20 residues of Tim23, normally accessible to the externally added protease in intact mitochondria, were found in a protease-protected location in mitochondria lacking Tim50 (22). We tested whether the presence of Tim50 perse is required for the exposure of Tim23 on the mitochondrial surface, or whether this process depends on the interaction of two proteins. In mitochondria having Tim23 double mutant, no clipping of Tim23 was observed even after prolonged exposure to PK (Fig. 5C). In contrast, in wild type mitochondria Tim23 was cleaved by externally added PK in a time-dependent manner. Taken together, these results demonstrate that the mutations that abolish the formation of the Tim23-Tim50 complex in vitro also affect their interaction in organelle and that this interaction is essential for viability of yeast cells and for exposure of Tim23 on the mitochondrial surface.

Finally, we analyzed the import of proteins into mitochondria isolated from cells carrying the Tim23Y70A,L71A mutation. Precursor proteins whose import is dependent on the TIM23 complex were imported into mitochondria with strongly reduced efficiency as compared with the wild type (Fig. 6). This was observed both for proteins targeted to the matrix, exemplified here by F1β and b2ΔDHFR, and to the inner membrane, exemplified by DLD(1–72)DHFR and Cox5a. In contrast, the import of proteins independent of the TIM23 complex, such as ATP/ADP carrier, was virtually unimpeached in mutant mitochondria. Also, mutant mitochondria had essentially the same membrane potential as the wild type, excluding secondary effects on import (Fig. 6F). In summary, these results demonstrate that the interaction between Tim23 and Tim50 is essential for efficient import by the TIM23 complex in both its translocation and insertion modes.

DISCUSSION

Tim23 is the central component of the TIM23 complex in which it performs a number of functions. Its C-terminal part is integrated in the inner membrane and is involved in the formation of the translocation channel that mediates the passage of unfolded precursor proteins through the inner membrane (1–3). The N-terminal hydrophilic region of yeast Tim23 is located in the intermembrane space and appears to have two functionally distinct segments. The first ~20 amino acid residues can span the outer membrane in a dynamic fashion,
actively responding to the translocation state of the complex (20, 28). The second part of the N-terminal domain serves several purposes. Early work demonstrated that it is involved in the membrane potential-dependent dimerization of Tim23 and in substrate recognition (29). More recent work showed that it interacts with the intermembrane space domain of Tim50 (21, 22, 30).

In the present study we established two independent assays to reconstitute the interaction between the intermembrane space domains of Tim23 and Tim50 in vitro using recombinant proteins. In addition, we identified residues that are essential for this interaction. The mutations of conserved residues Y70A and L71A on Tim23 abolished its interaction with Tim50 in vitro. Interestingly, these two residues map to the same region of Tim23 that was recently found by cross-linking to be in close proximity to Tim50 (24).

The results we obtained in in vitro experiments are in complete agreement with those obtained in vivo. Yeast cells carrying the double Y70A and L71A mutations in Tim23 were not able to grow at 37 °C, and the interaction between Tim23 and Tim50 was destabilized even at permissive temperatures. Intriguingly, destabilization of Tim23-Tim50 interaction did not lead to destabilization of Tim23 interaction with other components of the TIM23 complex, such as Tim17 and Tim44. This result supports the notion that the interaction between Tim50 and Tim23 is rather independent of the rest of the translocase (21–23).

The interaction between the intermembrane space domains of Tim23 and Tim50 is crucial for the function of the TIM23 complex. Mitochondria isolated from cells containing a mutant form of Tim23 imported TIM23 substrates with reduced efficiency as compared with wild type. Interestingly, both laterally sorted substrates and those completely translocated into the matrix. Mitochondria isolated from cells containing a mutant of Tim23 and Tim50 is crucial for the function of the TIM23 complex, such as Tim17 and Tim44. This result is in complete agreement with those obtained in vitro and in vivo experiments are in common agreement. Yeast cells carrying the double Y70A and L71A mutations in Tim23 were not able to grow at 37 °C, and the interaction between Tim23 and Tim50 was destabilized even at permissive temperatures. Intriguingly, destabilization of Tim23-Tim50 interaction did not lead to destabilization of Tim23 interaction with other components of the TIM23 complex, such as Tim17 and Tim44. This result supports the notion that the interaction between Tim50 and Tim23 is rather independent of the rest of the translocase (21–23).

The interaction between the intermembrane space domains of Tim23 and Tim50 is crucial for the function of the TIM23 complex. Mitochondria isolated from cells containing a mutant form of Tim23 imported TIM23 substrates with reduced efficiency as compared with wild type. Interestingly, both laterally sorted substrates and those completely translocated into the matrix were affected in their import. This demonstrates that Tim23 cooperates with Tim50 in both the translocation and insertion modes of the translocase.

The intermembrane space domains of Tim23 and Tim50 apparently form a minimal binding interface required for the interaction of these two proteins. However, it is questionable whether Tim23 and Tim50 interact solely through their soluble domains because we observed a relatively low $K_d$ for the interaction. Indeed, recent work suggested additional interaction surfaces involving the first transmembrane domain of Tim23 (24). In addition, the transmembrane domain of Tim50 appears to have a stabilizing role in the Tim23-Tim50 interaction.4

It is intriguing that the isolated intermembrane space domains of Tim23 and Tim50 interact with each other despite the fact that Tim23 is largely unfolded as shown by its CD spectrum. This could well reflect the actual folding state of this domain in vivo because its very N-terminal segment has the apparent ability to insert into and deinsert from the outer membrane in a precursor- and Tim50-dependent manner (Refs. 20 and 22 and this work) underlying the apparent necessity for a high conformational flexibility. Also, this domain was found to be extremely protease-sensitive in mitochondria with compromised outer membrane (28). Programs for the prediction of secondary structure failed to suggest major structural elements in the N-terminal domain of Tim23 (supplemental Fig. S2). Interestingly, the two amino acid residues we identified as contributing to the interaction of Tim23 with Tim50 are found in a region that is predicted to be folded into a short β-strand. Natively unstructured (or unfolded) regions were recognized as important mediators of interactions between partner proteins and suggested to undergo folding upon binding to their targets (31). It is therefore tempting to speculate that the substantial unstructured region of Tim23IMS folds into a native conformation upon its association with one of the other components of the mitochondrial protein import machinery or with substrate proteins. We propose that a conformational flexibility of Tim23 is required for its insertion into/deinsertion from the outer mitochondrial membrane, binding and transfer of precursor proteins through the translocation channel into the matrix, and triggering lateral opening of the channel in the case of precursors that are inserted into the inner membrane.

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