Membrane-embedded C-terminal Segment of Rat Mitochondrial TOM40 Constitutes Protein-conducting Pore with Enriched β-Structure*

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TOM40 is the central component of the preprotein translocase of the mitochondrial outer membrane (TOM complex). We purified recombinant rat TOM40 (rTOM40), which was refolded in Brij35 after solubilization from inclusion bodies by guanidine HCl. rTOM40 (i) consisted of a 63% β-sheet structure and (ii) bound a matrix-targeted preprotein with high affinity and partially translocated it into the rTOM40 pore. This partial translocation was inhibited by stabilization of the mature domain of the precursor. (iii) rTOM40 bound preprotein initially through ionic interactions, followed by salt-resistant non-ionic interactions, and (iv) exhibited presequence-sensitive, cation-specific channel activity. A pull-down assay and surface plasmon resonance (SPR) revealed that rTOM40 recognized preproteins by two distinct sequential interactions: initial ionic interactions, followed by salt-resistant non-ionic interactions, and (iv) exhibited presequence-sensitive, cation-specific channel activity. 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followed by salt-resistant hydrophobic interactions. These sequential interactions drove partial translocation of pre-proteins and sequestration of the mitochondrial processing peptidase (MPP)-processing site of pre-proteins within the rTOM40 pore. We then narrowed the location of the β-structure-enriched channel domain to the membrane-embedded C-terminal half of rTOM40 (residues 166–361). Purified recombinant rTOM40(N165) was in the oligomeric form of ∼170-kDa, as assessed by gel filtration, and exhibited both structural and preprotein-binding characteristics almost identical to those of rTOM40.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—For construction of the E. coli expression plasmid of rat TOM40, the 1086-bp CDNNA encoding N-terminal His-tagged TOM40 was cloned into the Ndel-BamHI sites of pET28a (Novagen) to obtain pET28a-NHis40 (14). The expression vector of rTOM40(N165) was prepared as PCR using pET28a-NHis40 as the template, and the following oligonucleotides as the primer: sense strand, 5′-GGGAAATTCATATGGGGAACGTGTTGGCTGCT-3′, and antisense strand, 5′-GCGGGATCCTCAGCCGATGGTGAGGCCAAA-3′. The Ndel and BamHI sites are underlined. The fragment was inserted into the Ndel-BamHI sites of pET28a to create pET28a-NHis40-(1–165). The DNA coding for N-terminal His6-tagged TOM40(N165) was prepared by PCR using pET28a-NHis40 as the template and the following oligonucleotides as the primer: sense strand, 5′-GGGAAATTCATATGGGGAACGTGTTGGCTGCT-3′, and antisense strand, 5′-GCGGGATCCTCAGCCGATGGTGAGGCCAAA-3′. The Ndel and BamHI sites are underlined. The amplified fragment was inserted between the Ndel-BamHI sites of pET28a to create pET28a-(1–165).

Purification of rTOM40, rTOM40(N165), and rTOM40-(1–165)—BL21(DE3)/LysE cells harboring the expression plasmids for rTOM40, rTOM40(N165), or rTOM40-(1–165) were cultured to A600 = 0.5, and protein expression was induced by 1 mM isopropyl 1-thio-galactopyranoside and cultured further for 3 h at 30 °C. The cells were suspended in the sonication buffer (50 mM Tris-HCl (pH 7.5) containing 250 mM NaCl, 1% Triton X-100, and 1 mM PMSF) and disrupted by sonication. For purification of rTOM40-(1–165), the soluble supernatant fraction obtained by ultracentrifugation was directly applied to a HitTrap chelating HP column (Amersham Biosciences). For purification of rTOM40 and rTOM40(N165), the sonicated cell debris was washed with sonication buffer containing 1% Triton X-100, and the unsolubilized fraction was recovered by centrifugation. This step was repeated nine times, and the unsolubilized materials were washed once with 20 mM Tris-HCl (pH 8.0) containing 500 mM NaCl, 4 M urea, and 1 mM 2-mercaptoethanol (2ME). After centrifugation, the unsolubilized fraction was recovered and analyzed by SDS-PAGE.

Measurement of Channel Activity—Electrical measurements were performed with nystatin-perforated patch recordings applied on lipid bilayer vesicles containing rTOM40. SCCC1–19 (10 μM) dissolved in the perfusate was applied using the Y-tube method, which allowed the external solution to be exchanged within 20 ms (16). The resistance between the patch pipette and the reference electrode in the external solution was 10–12 megohms. Ionic currents were measured with a patch clamp amplifier (EPC-7, List-Medical, Germany). All experiments were performed at room temperature. The composition of the pipette solution was 100 mM KCl, 50 mM potassium methane sulfonate, and 10 mM Hepes (pH 7.2). The external solution contained 150 mM KCl, 10 mM Hepes (pH 7.2), and 1 mM CaCl2. In some experiments, the reversal potentials were obtained as the membrane potential at which the current caused by the ramp voltage step of 30 ms to +100 mV with and without SCCC1–19 intersected with each other. The presence of the TOM40 pore was verified by measuring release of [14C]sucrose from the proteoliposomes containing rTOM40 or rTOM40(N165). The assay was carried out essentially as described by Zalman et al. (17) using [14C]sucrose and [3H]dextran (mean M, 70,000) as the permeable and impermeable substrates, respectively, except that rTOM40, rTOM40(N165), rTOM40-(1–165), or lactate dehydrogenase, in lieu of mitochondrial outer membrane, was reconstituted into asolectin liposomes. The reaction mixture was passed through a Sepharose CL-4B column (0.9 × 9.5 cm) equilibrated with 10 mM Hepes-KOH buffer (pH 7.4) containing 100 mM NaCl, 0.1 mM MgCl2, and 3 mM NaN3. The mixture was then dialyzed against 50 mM Tris-HCl (pH 7.5) containing 0.5% Brij35 and 20 μg of rTOM40 was added to the tube, vortexed, and incubated on ice for 4 h with intermittent mixing. After incubation, the reaction mixture was diluted in 50 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 50 mM sodium acetate. The reaction mixture was then dialyzed against 5 liters of 10 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl and 20 g of Bio-Beads SM-2 (Bio-Rad). The dialysate was concentrated using a membrane filter. Approximately 80% of the obtained proteoliposomes assumed unilamellar vesicles with ∼1 μm diameter.

Preparation of Mitochondria from HeLa Cells Expressing the Epitope-tagged rTOM40—HeLa cells were transfected with N-terminal His-tagged rTOM40 or C-terminal HA-tagged rTOM40 using FuGENE (Roche Applied Science) and cultured for 24 h. The cells were collected by centrifugation and homogenized in the homogenization buffer (10 mM Hepes-KOH buffer (pH 7.4), containing 0.22 mM mannitol, 0.07 mM sucrose, and 1 mM PMSF) by passing them through a 27-gauge needle 20 times using a syringe. The homogenate was centrifuged at 600 × g for 5 min, and the supernatant was then centrifuged at 6000 × g for 10 min to obtain the mitochondrial fraction.

Isolation of Proteoliposomes by Centrifugal Floatation—After reconstitution of proteoliposomes, the reaction mixture was adjusted to 1.6 mM sucrose, placed under the layers of 1.25 and 0.25 mM sucrose, and centrifuged in a Hitachi RP-S120AT3 rotor at 100,000 rpm for 90 min. Proteoliposomes floated to the 0.25 and 1.25 mM sucrose layers were recovered and analyzed by SDS-PAGE.

Binding Assay of Preproteins to rTOM40, rTOM40(N165), or rTOM40-(1–165)—Co-precipitation—The mixture containing 1 μg of Pd and the indicated amounts of rTOM40, rTOM40(N165), or rTOM40-(1–165) in 200 μl of the binding buffer (20 mM Hepes-KOH buffer (pH 7.4) containing 0.1% Brij35, and 50 mM NaCl) was incubated at 4 °C for 1 h. The reaction mixture was centrifuged at 45,000 rpm for 5 min. The obtained supernatant was mixed with 30 μl (50% slurry) of TALON metal affinity resin (Clontech) and incubated at 4 °C for 1 h. The beads were washed with the binding buffer, and the bound proteins were analyzed by SDS-PAGE and subsequent immunoblotting using anti-adrenodoxin IgG.

Protection of the Mpp-processing Site of Pd by rTOM40 or rTOM40(N165)—Binding of Pd to rTOM40, rTOM40(N165), or rTOM40-(1–165) and isolation of the complex by TALON beads were performed as described above. The isolated beads were suspended in 25 μl of the binding buffer, and the suspension was then incubated with 3 μg of yeast recombinant MPP in the presence of 2 mM MnCl2 at 30 °C for 30 min. The reaction was terminated with the sample loading buffer and analyzed by SDS-PAGE and subsequent immunoblotting using anti-adrenodoxin IgG.
CD Spectrum Measurement—CD spectra of rTOM40, rTOM40(N165), and rTOM40(1–165) were measured in 10 mM Tris-HCl buffer (pH 7.4) containing 0.5% Brij35 at 25 °C using a JASCO J-720 spectropolarimeter and a cuvette with a 1-mm light-path. Each spectrum represents an average of five scans from 195 to 250 nm at 0.1-nm intervals. The base line was established by subtracting the spectrum of the buffer alone. Analysis of the secondary structure was performed using the method of Reed and Reed (18).

Surface Plasmon Resonance Measurement—The SPR measurements were performed at 25 °C with a Bicore 3000 (Bicore AB). Purified rTOM40, rTOM40(N165), or rTOM40(1–165) was immobilized onto the sensor chip CM5 by amine-coupling according to the manufacturer’s protocol. Briefly, the coupling was performed in 10 mM sodium acetate buffer (pH 6.5) at a protein concentration of 0.5 μg/ml. The level of immobilization typically corresponded to 2000 resonance units, which corresponded to ≈2 ng of protein/mm² (Fig. 6, A–D). In Fig. 6E, rTOM40 was immobilized to the chip at 22,000 resonance units (corresponding to ≈22 ng of protein/mm²). Binding analyses were performed in 20 mM Hepes-KOH buffer (pH 7.4) containing 150 mM NaCl and 0.05% Brij35 (running buffer) at a flow rate of 20 μl/min. The sensor chip surface was regenerated by 50 mM HCl. Binding curves were analyzed using BIA-Evaluation software (version 3.2). The kinetic data fitting was performed using a Langmuir 1:1 binding model.

RESULTS

Purification of Rat TOM40—N-terminal His₆-tagged rTOM40 expressed in E. coli as inclusion bodies was solubilized by 6 M GdnHCl, applied to a Ni-NTA affinity column, and purified after dissociation of the purified TOM complex with 6 M GdnHCl, applied to a Ni-NTA affinity column, and rTOM40 expressed in E. coli. The purified rTOM40-(1–165) was eluted through a Superose 6 column with a peak at 250 kDa, although with a rather broad elution profile (see Fig. 5B).

Secondary Structure of rTOM40—A CD spectrum of rTOM40 in 5% Brij35 had a minimum value at 213 nm, crossover of the base line at 201 nm, and zero ellipticity at a wavelength at 235 nm (Fig. 1B). The secondary structure of rTOM40, estimated from the CD spectrum using the program of Reed and Reed (18) comprised 62.9% β-sheet, 10.0% α-helix, 5.9% turn, and 21.1% random structures in the protein. The content of the β-sheet structure of rTOM40 was comparable with that of recombinant Saccharomyces cerevisiae Tom40 (>60%) purified from inclusion bodies after solubilization with 8 M urea and either reconstituted into liposomes or solubilized in Mega9, although the CD spectra were distinct (12). The secondary structure of rTOM40 was significantly different from that of the oligomeric form of N. crassa Tom40, which was purified after dissociation of the purified TOM complex with octyl glucoside (less β-sheet, ~31%; more α-helix, 30%) (10).

Secondary Structure of rTOM40—A CD spectrum of rTOM40 expressed in E. coli as inclusion bodies was solubilized by 6 M GdnHCl, applied to a Ni-NTA affinity column, and rTOM40 expressed in E. coli. The purified rTOM40-(1–165) was eluted through a Superose 6 column with a peak at 250 kDa, although with a rather broad elution profile (see Fig. 5B).

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by the other interactions involving hydrophobic interactions. The so-called “cis-binding sites” or “cis-sites” of mitochondria or mitochondrial outer membranes, which are located in the protease-sensitive surface receptors Tom20 and Tom22, are sensitive to salt concentrations as low as 100 mM (22–26). Therefore, the “cis”-site of TOM40 involved in the initial precursor recognition binds preproteins through stronger ionic interactions than that for the cis-binding site of the mitochondrial outer membrane. Stan et al. (27) demonstrated that the isolated N. crassa TOM holocomplex and the proteinase K-treated core not only bind pSU9-DHFR but protect the precursor from cleavage by MPP, indicating partial translocation of the precursor protein into the TOM complex and that the MPP cleavage site is protected by the TOM complex against MPP. Because this MPP protection is a suitable criterion to assess Tom40 function, we examined MPP protection with rTOM40. Recombinant pAd was incubated with rTOM40 and then the pAd-rTOM40 complex was isolated using nickel resin, which was then subjected to MPP digestion. As shown in Fig. 3C, the pAd recovered as the complex with rTOM40 was protected against MPP, whereas unbound pAd was efficiently processed. This protection occurred as a function of the amount of rTOM40 (Fig. 3, D and E), and the reaction was essentially saturated by 4–8 μg of rTOM40 per 1 μg of pAd (roughly calculated, ~2–4 mol of rTOM40/mol of pAd, assuming the molecular size of rTOM40 and pAd to be 38 and 20 kDa, respectively). The rTOM40-dependent MPP protection was also observed for pSU9-DHFR (data not shown). Therefore, recombinant rTOM40 had properties similar to those of the isolated N. crassa TOM holocomplex or the proteinase K-treated core, which is composed solely of the oligomeric form of Tom40 (27). The TOM core complex was unable to partially translocate the preprotein unless phospholipids from the mitochondrial outer membrane were supplied externally (27). Because phospholipid P, was not detected in our rTOM40 preparation (data not shown), rTOM40 seemed to have folded correctly in Brij35 during the

![Fig. 3. Recognition of preprotein by purified rTOM40. A, binding of pAd to rTOM40 as measured by pull-down assay. pAd (1 μg) was incubated with the indicated amounts of rTOM40 (N-terminal His_6-tagged), and then the reaction mixtures were subjected to pull-down reaction by Ni-NTA beads. The recovered pAd-rTOM40 complex was subjected to SDS-PAGE followed by immunoblotting using anti-adrenodoxin antibodies and subsequent image analysis by a LAS1000 plus (Fuji Film Co.). The band intensities were calculated by setting the total pAd signal to 100% (shown in the right panel). B, salt sensitivity of the pAd-rTOM40 complex. The binding assay was performed as in A using 5 μg of rTOM40 in the presence of the indicated concentrations of NaCl. In the lower panel, the pAd-rTOM40 complex formed after 30 min of incubation was incubated with 500 mM NaCl (A') and then subjected to the pull-down assay as in A. In a separate experiment, pAd and rTOM40 were incubated in the presence of 500 mM NaCl (B) followed by the pull-down assay. The quantified results are shown in the right panel. C, sequestration of the MPP-processing site of pAd within the rTOM40 molecule. pAd (1.4 μg) and rTOM40 (3.8 μg) were incubated in 50 μl at 30 °C for 30 min. The reaction mixtures were then incubated with Ni-NTA beads to separate into pAd (unbound) and the pAd-rTOM40 complex (bound). Both fractions were then treated with (+) or without (−) MPP at 30 °C for 30 min, and the reaction mixtures were analyzed by SDS-PAGE and subsequent immunoblotting with anti-adrenodoxin antibodies. D, dose-dependent sequestration of the MPP-processing site of pAd by rTOM40. pAd (1 μg) and the indicated amounts of rTOM40 were incubated at 30 °C for 30 min. The reaction mixtures were then incubated with MPP at 30 °C for 30 min, which were subjected to SDS-PAGE and subsequent image analysis. The protection efficiency (%) was calculated as the ratio of pAd to pAd plus mAd, and shown in E.](image-url)
purification process to acquire the activity of partial translocation of preproteins even in the absence of phospholipids.

Domain Structure of \( \text{rTOM40} \) and Its Membrane Topology—
We then probed the domain structure of purified \( \text{rTOM40} \) using protease digestion. Elastase (2 \( \mu \mathrm{g/ml} \)) treatment of \( \text{rTOM40} \) at 0 °C for 30 min produced at least three distinct fragments (Fig. 4). Fragments 1 and 2 were formed transiently, and fragment 3 was formed stably. These fragments were detected even after 50 \( \mu \mathrm{g/ml} \) elastase digestion (see Fig. 4B). N-terminal amino acid sequencing revealed that fragments 1–3 had lost residues 1–7, 1–65, and 1–165, respectively. The antibodies against \( \text{rTOM40} \) (\( \alpha \)-TOM40) recognized only fragment 1, whereas two antibodies raised against the synthetic peptides corresponding to the regions near the C terminus (residues 323–345; peptide...
FIG. 5. Structural characteristics of purified rTOM40(ΔN165) and its properties of preprotein recognition. A, SDS-PAGE profile of purified rTOM40(ΔN165). B, elution profiles of rTOM40 and rTOM40(ΔN165) through Superose 6 column equilibrated with 20 mM Tris-HCl (pH 7.5) containing 0.5% Brij35 and 150 mM NaCl. C, CD spectrum of rTOM40(ΔN165). D, binding of pAd by rTOM40(ΔN165) as assessed by pull-down assay. pAd (1 μg) was incubated with the indicated amounts of rTOM40(ΔN165) (N-terminal His6-tagged), and then the reaction mixtures were subjected to pull-down reaction. Other conditions were as described in the legend to Fig. 3. E, salt sensitivity of the pAd-rTOM40(ΔN165) complex. The binding assay was performed as in D using 5 μg of rTOM40(ΔN165) in the presence of the indicated concentrations of NaCl. F, sequestration of the MPP-processing site of pAd within rTOM40(ΔN165) molecule. pAd and rTOM40(ΔN165) were incubated at 30 °C for 30 min. The reaction mixtures were then incubated with Ni-NTA beads. The pAd-rTOM40(ΔN165) complex was incubated with (+) or without (−) MPP at 30 °C for 30 min, and the reaction mixtures were analyzed by SDS-PAGE and subsequent immunoblotting with anti-adrenodoxin antibodies. In a separate experiment (ΔN165 = 0 μg), pAd was incubated with MPP at 30 °C for 30 min, and the reaction mixture was analyzed by SDS-PAGE and subsequent immunoblotting. The band intensities were quantified, and the processing efficiency (mAd/(pAd + mAd)) was calculated by setting the efficiency in the absence of rTOM40(ΔN165) to 100% (shown in the right panel). G, rTOM40(1–165) binds pAd but does not sequester the MPP-processing site within the molecule. The indicated amounts of N-terminal His6-tagged rTOM40(1–165) were incubated in 200 μl with 1 μg of pAd, and the reaction mixtures were subjected to the MPP protection assay as described in F.
40–1, and 189–207: peptide 40–2) recognized all three fragments (Fig. 4B). On the other hand, anti-His$_6$ antibodies only recognized the full-size rTOM40 (Fig. 4B). Thus, rTOM40 antibodies recognized the epitopes located within the N-terminal 65 residues of rTOM40. These results indicated that the C-terminal half of rTOM40 (residues 166–361; 21.4 kDa) folded...
to form a stable domain structure.

We then addressed the topology of rTOM40 in the mitochondrial outer membrane using antibodies against rTOM40 (α-TOM40) and against a synthetic peptide corresponding to residues 189–207 (α-peptide 40-2). Elastase treatment of rat liver mitochondria under isotonic conditions produced a fragment, which was recognized both by α-TOM40 and α-peptide 40-2 (Fig. 4C, left and middle panels). Under hypotonic conditions, elastase produced a fragment, which was detected only by α-peptide 40-2 (Fig. 4C, middle panel). From the size and reactivity with the antibodies, the bands produced under isotonic conditions and hypotonic conditions were considered to correspond to fragment 1 and fragment 2, respectively. These results indicated that the N-terminal site (Ala65–Ser68) of rTOM40 is exposed to the outer surface of the mitochondria, whereas the site Ala65–Ala66 is localized in the intermembrane space. The Ile165-His166 site, which is accessible to elastase in purified rTOM40 to produce fragment 3, was masked by the membrane or by the components of the TOM complex. The behavior of mitochondrial markers, mHsp70 (matrix protein), TIM23 (inner membrane protein extruding the N-terminal segment out of the inner membrane), and TOM20 (outer membrane protein extruding the bulk C-terminal portion to the cytosol), indicated that the protease digestion reactions were well controlled (Fig. 4C, right panel). Of note, fragments 1 and 2 were resistant to sodium carbonate (pH 11.5) extraction, indicating that they were firmly embedded in the membrane (data not shown). Topology of the N-terminal segment was further confirmed by using mitochondria isolated from HeLa cells expressing N-terminal His6-tagged rTOM40. As shown in Fig. 4D, the His6 epitope tag was removed by elastase treatment under isotonic conditions, indicating that the N-terminal segment of rTOM40 is exposed to the cytosol. We then probed the orientation of the C-terminal segment by using mitochondrial harboring C-terminal hemagglutinin (HA)-tagged rTOM40. When the mitochondria were treated with proteinase K under isotonic conditions, the HA tag was unaffected, whereas it was completely removed from rTOM40 when the outer membrane was ruptured by hypotonic treatment, thus indicating that the C-terminal segment is exposed to the intermembrane space (Fig. 4D, right panel). This was further confirmed by carboxypeptidase Y treatment. rTOM40 in the isolated mitochondria was resistant to carboxypeptidase Y treatment under isotonic conditions, whereas it was completely digested under hypotonic conditions or in the presence of Triton X-100 (Fig. 4E). As a control, rTOM22 that is inserted into the outer membrane in the N-α-Cα orientation exhibited the same susceptibility to the carboxypeptidase Y treatment (Fig. 4E). Taken together, rTOM40 is embedded in the outer membrane exposing its N-terminal segment to the cytosol and the C-terminal segment to the intermembrane space, whereas at least the site Ala65–Ala66 is exposed to the intermembrane space (Fig. 4F). Whether segment 1–165 is embedded in the membrane by a single or multispanning configuration remains to be determined. The predicted overall topology is distinct from that of N. crassa Tom40; the N- and C-terminal ends are exposed to the intermembrane space (28, 29). The N-terminal segment of S. cerevisiae Tom40 is exposed to the cytosol (12), but the topology of the C-terminal segment is not known.

Table I

| TOM40 | SCC 1–19 | pSU9-DHFR |
|-------|---------|-----------|
| WT (full) | $k_{a}$ (M$^{-1}$ s$^{-1}$) | 3.0 x 10$^{2}$ | 3.1 x 10$^{4}$ |
| $k_{d}$ (s$^{-1}$) | 8.7 x 10$^{-4}$ | 3.7 x 10$^{-6}$ |
| $K_{D}$ ($k_{a}$/$k_{d}$) | 3.0 x 10$^{-6}$ | 1.2 x 10$^{-10}$ |
| ΔN165 | $k_{a}$ (M$^{-1}$ s$^{-1}$) | 1.8 x 10$^{2}$ | 2.9 x 10$^{4}$ |
| $k_{d}$ (s$^{-1}$) | 8.7 x 10$^{-4}$ | 2.3 x 10$^{-5}$ |
| $K_{D}$ ($k_{a}$/$k_{d}$) | 4.9 x 10$^{-6}$ | 8.0 x 10$^{-10}$ |

rTOM40(ΔN165) has a Secondary Structure and Preprotein-binding Properties Comparable with rTOM40—Based on the above findings, we purified a recombinant protein rTOM40(ΔN165) (21.4 kDa) in which the N-terminal 165-residue segment of rTOM40 was deleted, essentially according to the procedure adopted for rTOM40 (Fig. 5A). On a Superose 6 gel filtration column, it was eluted at an apparent molecular size of ~170 kDa with a sharp elution peak compared with rTOM40 (Fig. 5B). The secondary structure calculated from the CD spectrum in 0.5% Brij35 (Fig. 5C) revealed 62.0% β-sheet, 1.8% α-helix, 1.8% turn, and 34.5% random structures.

rTOM40(ΔN165) had preprotein-binding properties comparable with those of rTOM40 as follows: (i) dose-dependent pAd binding (Fig. 5D); (ii) salt-sensitive initial binding of pAd, followed by salt-resistant binding (Fig. 5E); and (iii) sequestration of the MPP processing site within the rTOM40(ΔN165) pore (Fig. 5F). It should be noted that the purified recombinant form of segment 1–165 of rTOM40 (rTOM40(1–165)) bound pSU9-DHFR with a $K_{D}$ of 1.4 x 10$^{-10}$ M as assessed by SPR measurements (data not shown), but it failed to protect the precursor from attack by MPP (Fig. 5G), suggesting that the protection against MPP was because of specific interactions with rTOM40(ΔN165). Taken together, rTOM40(ΔN165) exhibited preprotein recognition properties as the import pore comparable with those of rTOM40. In support of these findings, rTOM40 and rTOM40(ΔN165) reconstituted into proteoliposomes exhibited permease activity for the vesicle-entrapped sucrose (see below).

Binding Kinetics of Preproteins to rTOM40 and rTOM40(ΔN165) as Analyzed by SPR—We next measured the kinetics of interaction of rTOM40 or rTOM40(ΔN165) with either the synthetic presequence SCC1–19 or recombinant pSU9-DHFR by using SPR. rTOM40 or rTOM40(ΔN165) was immobilized to the sensor tips, and various concentrations of presequence or pSU9-DHFR were injected. The binding curves obtained (Fig. 6 for rTOM40; data not shown for rTOM40(ΔN165)) were analyzed by using BIA Evaluation software. Calculated association ($k_{a}$) and dissociation ($k_{d}$) rate constants and $K_{D}$ values are summarized in Table I. The affinity of rTOM40 for presequence peptide SCC1–19 was 3.0 x 10$^{-6}$ M (Table I). No binding was observed with nonfunctional control peptides SCC1–19M (20) (Fig. 6B) and Syn2b (30) (data not shown). In contrast, however, rTOM40 exhibited ~10$^{-4}$-fold higher affinity (1.2 x 10$^{-10}$ M) for pSU9-DHFR compared with the synthetic presequence, suggesting that the mature segment of the precursor was responsible for the high affinity binding (Fig. 6C and Table I). When the conformation of the DHFR segment was stabilized with methotrexate, binding of pSU9-DHFR was strongly inhibited (Fig. 6D). Taken together, these results suggested that the affinity of rTOM40 for the presequence per se was rather low, and the affinity was greatly increased by the presence of the unfolded mature region of the preprotein.

We then examined the salt sensitivity of the interaction between rTOM40 and pSU9-DHFR. The interaction was salt-sensitive, and the binding was almost completely inhibited by 0.5 M NaCl (Fig. 6E), confirming the results of the pull-down assays (see Fig. 3). These results indicated that rTOM40 initially binds preproteins mainly through ionic interactions, which is followed by some other interactions including hydrophobic forces; the unfolded mature segment of preprotein seemed to contribute to the latter interactions (13). rTOM40(ΔN165) exhibited similar but slightly lower affinity
for SCC1–19 and pSU9-DHFR compared with full size rTOM40 (Table I and data not shown). The N-terminal 165 segment might contribute to stabilize the correct conformation of the pore-forming segment.

**rTOM40 and rTOM40(ΔN165) Exhibit Sucrose Passage Activity When Reconstituted into Liposomes**—Because our attempts to measure the channel activity for rTOM40(ΔN165) by using electrical methods were unsuccessful for technical reasons, we tried to measure the pore activity biochemically. Because the TOM complex, when reconstituted into proteoliposomes, mediates passage of small molecules (11, 31), we examined whether rTOM40(ΔN165) has sucrose passage activity, using the method adopted for measuring the activity of mitochondrial porin (17, 32). This assay measures the retention of large [3H]dextran (mean Mr, 70,000) versus small [14C]sucrose that had been trapped into proteoliposomes containing rTOM40 proteins by sieving through a Sepharose 4B column. These experiments revealed that both rTOM40 and rTOM40(ΔN165) mediated passage of sucrose to a significant extent (Fig. 7 and Table II). As the controls, heat-denatured rTOM40(ΔN165) and cytoplasmic enzyme lactate dehydrogenase were inactive in this assay. We thus concluded that the

![Image of Sucrose Passage Activity of rTOM40 and rTOM40(ΔN165)](image-url)

**Fig. 7. Sucrose passage activity of rTOM40 and rTOM40(ΔN165).** **A,** reconstituted proteoliposomes (asolecin +) or mock-treated proteins (asolecin −) were subjected to centrifugal floatation at 100,000 rpm for 90 min. The 0.25 and 1.25 m sucrose layers containing proteoliposomes were collected and analyzed by SDS-PAGE and Coomassie Brilliant Blue staining. As the control, mock-treated proteins remained in the bottom fractions of the tubes. **B,** elution profiles of the [14C]sucrose- and [3H]dextran-entrapped proteoliposomes. [14C]Sucrose and [3H]dextran were enclosed in the proteoliposomes reconstituted with asolecin and the indicated proteins. The reaction mixtures were subjected to gel filtration through Sepharose CL-4B. Radioactivities of the eluted fractions were measured. The [14C]/[3H] ratios of the peak fractions were normalized so that those of the incubation mixtures would be equal to 1.0. Other conditions were described under “Experimental Procedures.” **LDH,** lactate dehydrogenase.
C-terminal half-segment of rTOM40, like full-size rTOM40, mediates passage of small molecules across the membranes. These properties seem to correspond to the ability of rTOM40 and rTOM40(ΔN165) for sequestration of the MPP-processing site of preprotein within the molecule (see Fig. 3 and Fig. 5).

DISCUSSION

Virtually all the nuclear coded mitochondrial proteins are translocated and sorted into mitochondrial subcompartments via the TOM complex; preproteins transported to the inner compartments are translocated through the TOM channel irrespective of whether they are destined to soluble compartments or to the inner membrane. On the other hand, the outer membrane proteins are sorted by the TOM complex from the proteins destined for the inner compartments and anchored to the lipid bilayer of the outer membrane. As an initial step for understanding the mechanism of this diverse preprotein recognition by the TOM channel, we purified active recombinant rTOM40, and we analyzed the recognition properties using matrix-targeted preproteins.

Purified rTOM40 bound preproteins with high affinity and sequestered the MPP-processing site within the molecule. Furthermore, when reconstituted into liposomes, it exhibited presequence-sensitive cation-selective channel activity. Therefore, recombinant rTOM40 was correctly refolded to attain the functional conformation as the preprotein translocation pore. The CD spectrum of rTOM40 did not exhibit the light-scattering effects caused by aggregated species and revealed a greater than 60% β-sheet structure. This value coincided well with that for recombinant S. cerevisiae Tom40 (12), although the CD spectra differed considerably. In contrast, the β-sheet structure content of N. crassa Tom40 predicted by CD spectra or IR spectra was markedly lower with a maximum of 31% (10). The α-helical structure of rTOM40 (10%) was half that of N. crassa Tom40. The reason for the difference in the secondary structure between N. crassa and mammals is not known.

Most importantly, this study demonstrates that the purified membrane embedded C-terminal, half-formed ~170-kDa homo-oligomeric complex with a greater than 60% β-sheet structure and exhibited preprotein-binding properties comparable with those of rTOM40, suggesting that the C-terminal segment constitutes the preprotein conducting pore. Alignment of Tom40 proteins from several organisms revealed that the sequence conservation is higher in the C-terminal pore-forming segment compared with the N-terminal segment (14). Although attempts to measure the presequence-responsive channel activity of rTOM40(ΔN165) electrically were unsuccessful, we could demonstrate that it mediated passage of sucrose across the membrane. This preparation will help analyze the structure of the pore and preprotein recognition mechanisms.

rTOM40(ΔN165) was almost functionally identical with rTOM40 with respect to the preprotein recognition, and both exhibited enriched β-sheet structures, thus the β-barrel structure is responsible for the pore function as is the case for porin (28, 33). The β-structure content of rTOM40(ΔN165) was lower (62%) than that of rTOM40. Because the random coil structure was increased in rTOM40(ΔN165), proper refolding might be disturbed to some extent. The N-terminal 1–165 segment might be required for correct formation or stabilization of the pore structure, and this might be reflected in the decreased affinity of rTOM40(ΔN165) for pSU9-DHFR.

Here we demonstrated that purified recombinant rTOM40 and rTOM40(ΔN165) exhibited virtually identical properties with the TOM core complex (24, 27). They initially bind the preprotein through predominantly electrostatic interactions and partially translocate the preprotein to the salt-resistant trans-site that is inaccessible to MPP, probably within the translocation channel. Stabilization of the DHFR moiety by methylotrexate inhibited binding of pSU9-DHFR to rTOM40 or rTOM40(ΔN165), suggesting that the partial translocation is accompanied by unfolding of the mature segment, and the activity is restricted to the C-terminal half of rTOM40. These results also indicate that purified rTOM40 as well as rTOM40(ΔN165) contain the salt-sensitive cis-binding site. The salt-sensitive binding to the cis-site provided by the surface receptors Tom20 and Tom22 is much weaker than that in rTOM40 or rTOM40(ΔN165); cis-site binding of the preprotein was almost completely inhibited by 100 mM KCl (22–24). Consistent with this, the \( K_d \) values of preproteins for the cytoplasmic domain of import receptors Tom70 or Tom20 as measured by SPR were \( 10^{-7} \)–\( 10^{-8} \) M (34). This affinity difference might facilitate vectorial preprotein transfer from the surface import receptors to the cis-binding site of Tom40.

Analysis by SPR revealed that rTOM40 bound pSU9-DHFR with high affinity (in the \( 10^{-10} \) M range), and stabilization of the DHFR moiety greatly decreased the affinity. Most interestingly, rTOM40 bound a presequence peptide but with 10-fold lower affinity at 3.0 \( \times \) \( 10^{-6} \) M. These results indicate that the mature portion of the preprotein contributes significantly to the high affinity binding. It should be noted that the rTOM40+pSU9-DHFR complex or rTOM40(ΔN165)+pSU9-DHFR complex, once formed, was resistant to salt treatment, indicating a mode of interaction different from the initial interactions in the latter binding stage or in the trans-site binding in the purified molecules. The precise nature of the interaction of the preprotein with the trans-binding site remains to be determined. rTOM40 and rTOM40(ΔN165) thus possess...
virtually all the preprotein-binding properties characteristic of the TOM holocomplex.

What might be the function of the N-terminal 165-residue segment? rTOM40-(1–165) was expressed in E. coli as a soluble form. CD spectra of the purified recombinant rTOM40-(1–165) revealed that it has 49% α-helix, 6% β-sheet, and 45% random structures. The segment consisting of residues 1–65 should span the membrane at least once, although the exact states of membrane disposition of the segment including this and up to 165 residues remains unknown. Recombinant rTOM40-(1–165) bound preprotein with an affinity on the order of 10−10 M mainly through hydrophobic interactions; the complex was stable in the presence of 500 mM NaCl. Considering that purified rTOM40 initially binds preproteins by ionic interactions, these results suggest that the 1–165 segment functions in the later stages of preprotein translocation. In N. crassa Tom40, segment 41–60 (corresponds to residues 80–98 of rTOM40) is essential for proper assembly/stability of Tom40 in the TOM complex (35). In a recent report, residues 51–60 (correspond to residues 90–98 of rTOM40) and the C-terminal 3 residues (residues 321–323 which correspond to 353–355 of rTOM40) are required for assembly beyond the 250-kDa assembly intermediate of the TOM complex (36). Thus, the N-terminal 1–165 segment might also be involved in the assembly with the TOM components such as Tom22 and small Tom proteins or function as the interface of releasing outer membrane proteins from the import pore into the lipid bilayer. Another possibility is that the N-terminal 1–165 segment is required for coupling the TOM complex with the translocation of inner membrane complex during preprotein translocation from the outer membrane to the inner membrane.

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