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Structural elements of the mitochondrial preprotein-conducting channel Tom40 dissolved by bioinformatics and mass spectrometry

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ABSTRACT

Most mitochondrial proteins are imported into mitochondria from the cytosolic compartment. Proteins destined for the outer or inner membrane, the inter-membrane space, or the matrix are recognized and translocated by the TOM machinery containing the specialized protein import channel Tom40. The latter is a protein with β-barrel shape, which is suggested to have evolved from a porin-type protein. To obtain structural insights in the absence of a crystal structure the membrane topology of Tom40 from *Neurospora crassa* was determined by limited proteolysis combined with mass spectrometry. The results were interpreted on the basis of a structural model that has been generated for NcTom40 by using the structure of mouse VDAC-1 as a template and amino acid sequence information of ~270 different Tom40 and ~480 VDAC amino acid sequences for refinement. The model largely explains the observed accessible cleavage sites and serves as a structural basis for the investigation of physicochemical properties of the ensemble of our Tom40 sequence data set. By this means we discovered two conserved polar slides in the pore interior. One is possibly involved in the positioning of a pore-inserted helix; the other one might be important for mitochondrial pre-sequence peptide binding as it is only present in Tom40 but not in VDAC proteins. The outer surface of the Tom40 barrel reveals two conserved amino acid clusters. They may be involved in binding other components of the TOM complex or bridging components of the TIM machinery of the mitochondrial inner membrane.

1. Introduction

Channeling of proteins across biological membranes through nanometer-scale pores is a process common to all living cells [1,2]. In all identified pathways the transfer of proteins across the lipid bilayer requires an assembly of proteins, which recognize the polypeptide to be translocated, mediate its insertion into a pore, facilitate the transfer through the pore, and drive the movement so that the transport becomes vectorial. In outer membranes of mitochondria, the translocon at the outer membrane of mitochondria (TOM) is such a molecular machine evolved for this function [3–6]. Within the TOM complex, Tom40 represents the pore for transport of virtually all cytosolic proteins destined for mitochondria. It forms a cation-selective, hydrophilic channel [7–11], which binds to mitochondrial pre-sequences [12,13] and facilitates translocation of unfolded or partially folded proteins from the cytosol to the inter-membrane space of mitochondria [14,15]. Tom40 is an essential component of the TOM complex and already moderate reduction of the Tom40 protein content shows a drastic reduction of precursor protein import manifesting the central role of this protein in translocation [16]. In addition, specific mutations of the Tom40 amino acid sequence resulted in an ineffective transfer of preproteins to the mitochondrial inner membrane protein translocase TIM23 [17]. All results together indicate that the Tom40 channel is not a passive pore, but plays an active role in protein sorting for all sub-mitochondrial locations.

The secondary structure of Tom40 from several organisms has been investigated by CD and FTIR spectroscopy. These studies revealed an enriched β-sheet secondary structure, similar to the mitochondrial voltage-dependent anion channel VDAC [10,18–20]. Consistently, a β-barrel conformation was postulated early on [21,22]. Recent modeling of the Tom40 structure based on the mouse VDAC-1 [23] is consistent with the existence of 19 β-strands and one N-terminal α-helical segment located inside the pore [24–26].

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It was demonstrated that precursor proteins can be chemically cross-linked to the *cis* and *trans* site of Tom40 [12]. This interaction is speculated to be essential to prevent precursor protein aggregation [13]. A subsequent mapping of the interaction sites within the presequence revealed that Tom40 is in close contact with the central region of the presequence while the N-terminus is bound to the intermembrane space domain of Tom22 [27]. In Tom22 mutants interactions between the N-terminus of the pre-sequence and Tom40 are observed [27]. Remarkably, by a similar strategy using full length protein fused to DHFR cross-links between Tom40 and the mature domain but not with the presequence were observed [27]. Thus, a specific binding motif within pre-sequences for the interaction with Tom40, which can only be transient, has not yet been identified. Based on this finding it is tempting to assume that the mode of interaction between Tom40 and precursor proteins parallels the mode of chaperone-substrate interaction by exhibiting a specific surface for the recognition of hydrophobic or polar stretches [28].

The analysis of purified Tom40 protein and of the TOM core complex after reconstitution into planar lipid bilayers indicated that Tom40 forms a cation selective membrane pore with an estimated inner pore diameter of 20 to 25 Å [7,10,19,29,30]. At high voltages, the channel revealed a complex gating behavior indicating fluctuation between different conformational states [7,19,31]. As expected, mitochondrial pre-sequence peptides were significantly more potent in blocking the pore than the synthetic non-mitochondrial model peptides [32].

In summary, Tom40 engages multiple interactions with precursor proteins and TOM complex components. Inspired by the solved structure of VDAC, several homology models were generated [24–26]. Remarkably, however, they could not yet explain the multitude of properties observed by biochemical and biophysical means. Here, we aimed at a structural description of the protein topology and its support by biochemical studies. We built a homology model using a consensus alignment approach to construct a multiple sequence alignment from a non-redundant set of ~270 Tom40 and ~480 VDAC amino acid sequences. Our alignment approach allowed detecting homologous secondary structure elements of these two divergent but phylogenetically related protein families. The structural architecture is displayed for Tom40 from *Neurospora crassa* (NcTom40) and the model is supported by peptide-bond accessibility probed by enzymatic hydrolysis. Mapping the properties of the ensemble of Tom40 sequences in our data set onto the model reveals conserved amino acid residues out- and inside the pore, which may play key roles in the assembly of the protein translocation machinery TOM and the transport of precursors, respectively. Based on the model we can reconcile many of the established results.

2. Material and methods

2.1. Purification and preparation of NcTom40 for proteolysis

Tom40 protein was purified from mitochondria of *N. crassa* strain GR-107 that comprises a hexahistidinyl-tagged form of Tom22. Purification was conducted accordingly as previously described [10,31,33] with minor modifications regarding the buffers. Mitochondria were solubilized in 1% (w/v) n-dodecyl-β-maltoside (DDM), 20 mM Tris-HCl pH 8.5, 20% (v/v) glycerol, 300 mM NaCl, 20 mM imidazole and 1 mM PMSF for 30 min at 4 °C. By applying an ultracentrifugation step (181,000×*g*, 40 min) extract was clarified and further bound to a nickel-nitriloacetic acid column (GE Healthcare). The column was washed with 0.1% (w/v) DDM, 20 mM Tris–HCl pH 8.5, 10% (v/v) glycerol, 300 mM NaCl, 1 mM PMSF, before Tom40 was eluted with 3% (w/v) n-octyl-β-D-glucopyranoside (OG, Glycon), 20 mM Tris–HCl pH 8.5, 2% (v/v) DMSO or 10% (v/v) glycerol. Peak fractions of OG elution step were pooled and concentrated with a 50 kDa MWCO filter (Sartorius) to a final protein concentration of 2 to 10 mg/ml. Concentrated protein sample was further dialysed (6–8 kDa MWCO; Spectrum Laboratories, Inc.) against 100× the volume of OG elution buffer at 4 °C overnight by stirring. Final sample was then diluted with OG elution buffer to a final protein concentration of 1 mg/ml for proteolysis and stored at 4 °C. Protein purity was examined by sodium dodecyl sulfate gel electrophoresis and staining with Coomassie Brilliant Blue. Determination of protein concentration was carried out via UV absorbance spectroscopy at 280 nm with a NanoDrop ND-1000 spectrophotometer (Thermo Fischer Scientific). The protein concentration was then calculated using an extinction coefficient of 35410 M^{-1} cm^{-1} estimated by primary sequence analyses of NcTom40 via ProtParam on the EXPASY.org proteomics server [34,35] with the tyrosine and tryptophan extinction coefficients of Pace et al. [36].

2.2. Proteolysis

Proteolysis of NcTom40 was performed *in vitro* on the basis of previous methods [37]. 2 µg of NcTom40 in 50 µl of 3% (w/v) n-octyl-β-D-glucopyranoside, 20 mM Tris–HCl pH 8.5 and 2% (v/v) DMSO were incubated with either 0.2 µg Asp-N, Lys-C, Glu-C or Trypsin at a protein ratio protease of 1:10 (w/w) at 25 °C. 50 µl aliquots for SDS-PAGE analysis were taken after 10 s, 1 min, 5 min, 15 min and 30 min. 10 µl aliquots for mass spectrometry analysis were taken after 1, 10, 60 and 240 min. Digests were stopped by acidifying the aliquots with 1 µl of 10% TFA and putting them on ice. For ESI-MS/MS experiments samples were passed through 10 kDa cut off Nanosep filtration units (Pall, Ann Arbor, United States) to remove undigested protein and part of the detergent.

2.3. Mass spectrometry

Peptide mass fingerprints and MALDI-TOF-TOF spectra of NcTom40 digests were acquired on an Autoflex III MALDI-TOF-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). The instrument was operated in the positive ion mode and externally calibrated using peptide calibration standards (Bruker Daltonics, Bremen, Germany). NcTom40 protein digests were desalted and concentrated using micro-C_{18} ZipTips (Millipore, Schwalbach, Germany) according to the manufacturer's protocols. Peptides were eluted directly onto a stainless steel target using a HCCA (α-Cyano-4-hydroxycinnamic acid) matrix solution (5 mg/ml in 50% ACN/ 50% 0.1% TFA, v/v). Peptide mass fingerprint data and MS/MS spectra were recorded in reflector mode using an accelerating voltage of 21 kV. Peptide mass fingerprint spectra were analyzed in the MS mode with 2000 laser shots per sample to ensure good S/N quality. MS/MS analysis was done with varying number of laser shots. Flex Analysis 3.0 and Bio-Tools 3.0 software (Bruker Daltonics, Bremen, Germany) were used for data processing.

Nano-LC-ESI-MS/MS experiments were performed on an ACQUITY nano-UPLC system (Waters, Milford, USA) directly coupled to a LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Fisher, Bremen, Germany). Digests of NcTom40 were concentrated and desalted on a precolumn (2 cm × 180 µm, Symmetry C_{18}, 5 µm particle size, Waters, Milford, USA) and separated on a 20 cm × 75 µm BEH 130 C_{18} reversed phase column (1.7 µm particle size, Waters, Milford, USA). Gradient elution was performed from 1% ACN to 50% ACN in 0.1% FA within 1 h. The LTQ-Orbitrap XL was operated under the control of XCalibur 2.0.7. software. Survey spectra ($m/z = 250–1800$) were detected in the Orbitrap at a resolution of 60.000 at $m/z = 400$. Data-dependent tandem mass spectra were generated for the five most abundant peptide precursors in the linear ion trap. For all measurements using the Orbitrap Detector internal calibration was performed using lock-mass ions from ambient air.

Mascot™ 2.2 software (Matrix Science, London, UK) was used for protein identification from MALDI-TOF and ESI-MS/MS experiments.