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Improving the Resistance of a Eukaryotic β -Barrel Protein to Thermal and Chemical Perturbation

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Abstract

Beta-barrel membrane proteins have regular structures with extensive hydrogen bonding networks between their transmembrane (TM) α -strands, which stabilize their protein fold. Nevertheless, weakly stable TM regions exist, which are important for the protein function and interaction with other proteins. Here, we report on the apparent stability of human Tom40A, a member of the 'mitochondrial porin family' and main constituent of the mitochondrial protein-conducting channel TOM. Using a physical interaction model TmSIP for α -barrel membrane proteins, we have identified three α -strands unfavorable in the TM domain of the protein. Substitution of key residues inside these strands with hydrophobic amino acids results in a decreased sensitivity of the protein to chemical and/or thermal denaturation. The apparent melting temperature observed when denatured at a rate of one degree per minute, is shifted from 73 to 84 °C. Moreover, the sensitivity of the protein to denaturant agents is significantly lowered. Further, we find a reduced tendency for the mutated protein to form dimers. We propose that the identified weakly stable α -strands 1, 2 and 9 of human Tom40A play an important role in quaternary protein-protein interactions within the mammalian TOM machinery. Our results show that the use of empirical energy functions to model the apparent stability of α -barrel membrane proteins may be a useful tool in the field of nanopore bioengineering.

Keywords

Eukaryotic porins; bacterial porins; human Tom40; TOM complex; oligomerization states; weakly stable transmembrane α -strands

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INTRODUCTION

The transmembrane (TM) domains of β -barrel membrane proteins have regular structures with an extensive hydrogen bonding network between the individual β -strands¹². Despite this strong network, unfavorable or weakly stable regions exist in several β -barrel membrane proteins^{3; 4}. They are often important for their function, such as voltage sensing⁵, flux control of metabolites and ion-sensing (see⁶⁷ for a detailed review).

In general, β -barrel membrane proteins are stabilized through binding of β -helices to weakly stable regions inside or outside of the pore, so-called in-plugs or out-clamps, through formation of oligomers via protein-protein interfaces, or interactions with lipids^{3; 4; 6; 8}.

In this work, we explore whether the prediction of weakly stable regions in bacterial β -barrels^{3; 4} can be adapted to human Tom40A (hTom40A) and reveal further insights on the structural organization of this protein.

In eukaryotes, Tom40 proteins represent an essential class of pore proteins, which facilitate the translocation of unfolded proteins from the cytosol into mitochondria. They comprise the main subunit of the TOM import machinery in mitochondrial outer membranes^{9; 10; 11; 12; 13; 14}.

It is generally predicted that all eukaryotic Tom40 proteins belong to the ‘mitochondrial porin’ superfamily. Thus, hTom40A most likely constitutes a β -barrel architecture similar to VDAC-1 with 19 β -strands and a short β -helix located inside the pore^{15; 16; 17}. Consistent with this model, circular dichroism and Fourier transform infrared secondary structure analyses of Tom40 and VDAC proteins from different organisms revealed a dominant β -sheet structure with a small β -helical part^{9; 18; 19}.

In the present study, we calculated the energy of each amino acid in the predicted native conformation of hTom40A using a recently introduced empirical potential function, which was developed based on extensive combinatorial analysis of known bacterial β -barrel membrane protein structures³. We have identified three β -strands, 1, 2 and 9, in the TM domain of hTom40A that contribute to the overall sensitivity of the protein to denaturation. We show that mutagenesis of the predicted specific destabilizing amino acid residues within these strands leads to a higher resistance to thermal or chemical perturbation of the barrel. Similar results were obtained with Tom40 from *Aspergillus fumigatus* (AfTom40). We propose that the unstable β -strands 1, 2 and 9 of the TM domain of hTom40A interact with other Tom40 molecules or subunits of the TOM complex.

RESULTS

Weakly stable regions in hTom40A and oligomerization index

The stability of β -barrel membrane proteins is determined by the balance between favourable hydrogen bonding, van der Waals and hydrophobic interactions as well as unfavourable conformational entropy. To identify weakly stable regions in the TM domain of wild-type hTom40A (Fig. 1A and B), we estimated the energetic contribution of all amino acid residues to the β -strand stability of the protein by using a computational approach³ that has recently been applied to model the conformational stability of 25 non-homologous β -barrel membrane proteins of known structure. Briefly, we calculated energetics of embedding specific residue types at different regions of the TM domain, the stabilizing effect due to interactions between residues on neighbouring strands through strong H-bond, weak H-bond, and side-chain side-chain interactions using the updated TmSIP empirical energy parameters^{3; 20}.

We calculated the contribution of residues to the empirical energy for each α -strand (Fig. 1C). Strands 1, 2 and 9 have significantly higher empirical energies and are thus less favorable than the rest of the protein. Then, the oligomerization index Q_{wt} , which summarizes the energy deviation of unstable strands from the expected energy value for all the strands in the protein, was calculated to be $Q_{wt} = 2.48$. This is based on the observation that highly unfavourable strands are often associated with protein-protein interfaces in the TM region.

In data sets using sequence information of bacterial α -barrel membrane proteins, a protein can be predicted to be monomeric if the oligomerization index is < 2.25 and oligomeric if it is > 2.75 ³. The oligomerization index of hTom40A is between these prediction thresholds. Theoretically, wild-type hTom40A may thus exist as stable monomers but may also form higher order complexes through distinct protein-protein interaction interfaces.

We further examined the contribution of all amino acids facing the membrane lipids within the predicted three unfavorable strands 1, 2 and 9 (Fig. 1D). In this analysis, residues K107 in strand 1, H117 in strand 2, and H220 in strand 9 were found to contribute the most to the instability of these strands. Based on our calculations, we predict strands 1, 2 and 9 of hTom40A to be unfavorable / weakly stable. Each strand can have two orientations; the side-chain of the first residue can either face the lipid environment or the internal of the barrel. The orientations of strands are predicted using the energy scale in reference³ such that the number of costly burial of ionizable/polar residues facing the lipid environment is overall minimized.

Secondary and tertiary structure of hTom40A

In order to account for the differences between bacterial and mitochondrial α -barrel membrane proteins, and keeping in mind that hTom40A forms complexes also with other components of the TOM machinery, we wanted to construct a mutant protein to have an oligomerization index Q below 1.5 so that the resulting mutant hTom40A would form more stable monomers, possibly without protein-protein interaction interfaces.

To test to what extent α -strands 1, 2, and 9 determine the overall resistance to denaturation and oligomerization state of hTom40A, we designed five mutants, termed K107L, H117L, H220L, K107L/H117L and K107L/H117L/H220L, where residues K107, H117 and H220 of hTom40A are replaced by leucines. Leucine is predicted to be the most stabilizing amino acid when facing the lipid within the core region of a transmembrane α -strand³.

The empirical energy profile of the triple mutant hTom40A^{mut} (K107L/H117L/H220L) is shown in Fig. 1E. The oligomerization index of this mutant ($Q_{K107L/H117L/H220L} = 1.37$) was significantly lower than that of the wild-type protein ($Q_{wt} = 2.48$), predicting a very robust monomeric α -barrel membrane protein. Energy calculations of single and double mutants revealed higher indices ($Q_{K107L} = 2.08$, $Q_{H117L} = 2.01$, $Q_{H220L} = 2.03$, $Q_{K107L/H117L} = 1.91$, $Q_{K107L/H220L} = 1.71$, $Q_{H117L/H220L} = 1.68$) predicting less stable proteins.

Mammalian Tom40A isoforms show a very high sequence identity amongst each other ($>91\%$, data not shown). All include a remarkable N-terminal poly-proline region, which is only present in mammalian Tom40A. So far, thermal stability analyses of the TM α -barrels of mammalian full length Tom40A proteins using UV-CD spectroscopy have not been successful in our hands (data not shown). The α -barrel UV-CD signals appeared to be superimposed by strong CD signals caused by the poly-proline rich region of the protein. As a consequence of this interference, temperature-induced transitions of the α -barrel itself could not be monitored accurately. Since the N-terminal poly-proline rich domain in mammalian Tom40A proteins has no effect on the channel formation²¹, we deleted the

poly-proline region of all hTom40A proteins to improve the CD-signal in heat-induced unfolding measurements.

Wild-type and mutant Tom40A proteins were expressed in *E. coli* and Nickel-affinity purified from inclusion bodies under denaturing conditions. The proteins were refolded by rapid dilution of denaturant into LDAO-containing detergent buffer and further purified via size exclusion chromatography. Analysis of refolded proteins by SDS-PAGE and Coomassie and silver staining (Fig. 2A) indicated that all isoforms were virtually pure. UV-CD spectroscopy showed typical beta barrel spectra for wild-type and mutant proteins with similar curves for all hTom40A isoforms^{9; 10; 18; 21} (Fig. 2B). The spectral characteristics of the wild-type hTom40A and the triple mutant are summarized in supplementary Table 2. At wavelengths >250 nm, the CD spectra approached ellipticity values close to zero, indicating that the protein preparations were virtually free of higher order aggregates, which would cause light-scattering effects and interfere with the interpretation of the data.

Further, tertiary structure was analyzed via tryptophan fluorescence spectroscopy. The emission spectra of wild-type and all mutant Tom40 proteins (Fig. 2C) were exactly the same, with intensity maxima at approximately 340 nm and an unchanged width of the emission spectra. This can be interpreted as an unaltered environment of the widely spread tryptophan residues, W188, W259 and W322, which are conserved in the amino acid sequence of all isoforms and remain unchanged in all our designed mutant proteins. In summary, we suggest that secondary and tertiary structures match in all hTom40A isoforms and consequently differences in resistance to chemical and thermal perturbation and oligomerization state are not due to an altered protein structure.

Thermal and solvent stability of hTom40A

To compare the thermal stability of wild-type and mutant hTom40A, CD signals were measured at different temperatures at constant wavelength. Wild-type hTom40A unfolded at an apparent melting temperature of about 73 °C, when denatured at a rate of 1 °C per minute (Fig. 3). In line with our energy calculations described above, hTom40A with substitutions at positions K107, H117 and H220 revealed an apparent midpoint of resistance to thermal denaturation of approximately 84 °C (Fig. 3). The single and double mutants did not appear to be significantly more stable than wild-type protein, however differences proved not to be significant (data not shown).

To provide further evidence that substitution of unfavorable amino acids in the TM domain of hTom40A results in conformational stabilization of the protein, we compared tryptophan fluorescence spectra of wild-type and mutated hTom40A in the presence of chemical denaturants. The change in tryptophan fluorescence of wild-type and mutated hTom40A, respectively, was monitored at different Guanidine hydrochloride (GnHCl) concentrations, (Figs. 4A and B). Wild-type Tom40 was completely denatured in ~4.5 M GnHCl. The apparent midpoint of unfolding occurred at a concentration of ~3.1 M GnHCl. On the other hand, the resistance of mutant Tom40 K107L/H117L/H220L to chemical denaturation was greatly enhanced (Fig. 4C). The apparent midpoint of unfolding of hTom40A was shifted from 3.1 to approximately 4.8 M GnHCl. Mutant hTom40A K107L/H117L/H220L completely unfolded at ~6.3 M GnHCl. These results were obtained no matter whether unfolded or refolded Tom40 was incubated with GnHCl (Figs. 4C and D), showing the reversibility of the chemical denaturation.

Chemical unfolding of hTom40A K107L, H220L, H117L and K107L/H117L occurred at around 2.9, 3.5, 3.9 and 4.2 M GnHCl (Fig. 4C). These mutants were thus more resistant to chemical denaturation than wild-type hTom40A but less resistant than the triple mutant K107L/H117L/H220L. Our data indicate that the resistance of the mutants to chemical

denaturation increased with decreasing q -values. They furthermore suggest that wild-type hTom40A unfolds via a multi-state mechanism whereas unfolding of “stabilized” protein follows a two-state conformational transition.

Thermal and solvent stability of AfTom40

To further test the prediction of weakly stable β -strands in eukaryotic porins, we applied stability calculations to full length Tom40 from *Aspergillus fumigatus* (AfTom40). Comparison of full length AfTom40 and wild type hTom40A 1-82 revealed a moderate identity at the amino acid level (~25% identical residues in the conserved TM part, including the N-terminal helix), but a similar β -barrel secondary structure (supplementary Fig. 1). We identified five unstable regions in AfTom40 to compromise the stability of β -strands 1, 5-7, 9, 13 and 17 (Fig. 5A). The q -value of the wild-type protein was calculated to be 2.81. To see to what extent these β -strands determine the stability of the protein, an AfTom40 variant was made with mutations K69H, N150H, S180L, S236A and K302H. These mutated residues were predicted to be the most favorable residues in the respective positions of the β -strand by the TmSIP potential function. The q -index of this mutant was calculated as 1.66 (Fig. 5A). Wild-type and mutant AfTom40 were over-expressed in *E. coli* cells, purified under denaturing conditions and refolded into polyoxyethylene monolauryl ether (Brij35) detergent containing buffer in a similar way as hTom40A (Fig. 5B). Far UV-CD spectra of both proteins (Fig. 5C) showed a β -barrel fold as for hTom40A. Tryptophane fluorescence emission spectra were also almost identical supporting a similar structure for both AfTom40 proteins (Fig. 5D). Thermal denaturation of both AfTom40 proteins followed by CD-spectroscopy revealed no difference in the apparent melting temperature but a clear change in the cooperativity in unfolding (Fig. 5E). In contrast to thermally induced unfolding, chemically induced unfolding of wild-type and mutant AfTom40 proteins revealed an apparent stabilization of the mutant protein (Fig. 5F). The apparent midpoint of unfolding of wild-type protein was observed around 2.5 M and was shifted to about 3.2 M GnHCl in the mutant protein. Both patterns of unfolding were in line with those observed for thermal unfolding (Fig. 5E) and those observed for hTom40A (Fig. 4C, D).

Oligomerization states of hTom40A

Previous studies revealed that weakly unfavorable regions in bacterial β -barrel proteins can be stabilized through oligomerization of monomers³. Our energy calculations described above predicted a very stable monomeric form for the mutant hTom40A K107L/H117L/H220L. To verify this hypothesis, we conducted chemical cross-linking experiments on both wild-type and triple-mutant Tom40 solubilized in LDAO. Both protein samples were incubated with glutaraldehyde, which forms covalent bonds between primary amine groups. The degree of intermolecular cross-link formation was evaluated by SDS-PAGE and Western blotting (Fig. 6). Wild-type hTom40A was present mostly in its monomeric form, but also formed dimers. In contrast, mutant protein was predominantly monomeric and revealed virtually no dimers. Similar results were obtained with proteins in β -dodecylmaltoside-containing buffer (data not shown).

It should be noted, however, that long exposures of both proteins to glutaraldehyde resulted in protein aggregates which did not enter the SDS gel. In summary, we conclude successful stabilization of the hTom40A β -barrel. Hence, for mutant hTom40A K107L/H117L/H220L oligomerization is not required to compensate for unfavorable regions.

DISCUSSION

In this study, we applied a recently developed method for the prediction of unfavorable regions in bacterial β -barrel membrane proteins³ to eukaryotic hTom40A. We identified and

experimentally confirmed two regions in the TM domain of the protein (α -strands 1 and 2, and α -strand 9), which have strong impact on its resistance to thermal and chemical perturbation. Oligomerization of hTom40A monomers might provide the mechanism to counterbalance the overall α -barrel instability, indicating possible protein-protein interfaces in mammalian Tom40 proteins. Indeed, existence of dimeric and trimeric Tom40 forms was shown for fungal Tom40^{27; 28}. However, for mammalian Tom40 proteins experimental evidence has yet to be shown. Nevertheless, our results suggest that α -strands 1, 2 and 9 in the TM domain of mammalian Tom40 might be important for the association of single Tom40 molecules. In addition, other subunits of the mitochondrial protein import machinery TOM, e.g. the small Tom proteins, might also interact with α -strands 1, 2 and 9 in the TOM complex from mammals²⁹.

Interestingly, the key amino acids K107, H117 and H220 in the two unfavourable regions of hTom40A are not strongly conserved between different species (e.g. fungi and plants). This and the results of our stability calculations for AfTom40 indicate that weakly stable regions in mammalian Tom40 proteins differ from evolutionarily more distant Tom40 α -barrels.

Using the above described method for the prediction of unfavourable regions on fungal Tom40 from *Aspergillus fumigatus*, we identified five unstable domains comprising strands 1, 5-7, 9, 13 and 17. As in the case of mammalian TOM complex, these five unstable domains in fungal Tom40 proteins might play an important role for the interaction with other subunits of the TOM complex in fungi³³. It is tempting to suggest that the assembly and dissociation of the TOM complex is caused by the compensation of weakly stable regions in the TM domain of Tom40 by the association of other TOM subunits and Tom40 itself, not only in detergent buffer but also in the *in vivo* membrane environment of mitochondria.

Empirical energy calculations on AfTom40 predicted a significantly more favourable interactions of the protein upon mutations in α -strands 1, 7, 9, 13 and 17. However, experimental analysis revealed only a moderate resistance of the protein to chemical and thermal denaturation. A possible explanation for this could be that the full length AfTom40 construct used in this study comprises not only a α -barrel domain, but has additional large N- and C-terminal random coil and α -helical tails. They unfold at low temperatures and low GdnHCl concentrations compromising the CD- and fluorescence signals of α -barrel unfolding and complicate the empirical energy calculations of the α -barrel. Another reason could be the imprecise nature of the identified locations of the 19 α -strands of AfTom40, which is used for the empirical energy calculations.

We conclude that computational prediction of unfavourable regions in α -barrel membrane proteins displays a useful tool to improve the resistance of eukaryotic α -barrel proteins to thermal and chemical denaturation. With bioinformatics-derived empirical energy parameters and calculation of thermodynamic properties based on a firm statistical mechanical model, we can now begin to combine both experimental and computational approaches to engineer α -barrel membrane proteins with rationally designed biophysical properties. We propose that our method can be easily adapted to the stabilization of other α -barrel membrane proteins, which is of high interest in e.g. nanotechnology³⁰ and structural biology^{31; 32}.

MATERIALS AND METHODS

Secondary structure and weakly stable amino acid residues prediction

The software packages PRED-TMBB³⁴ and TMBETAPRED-RBF³⁵ were used for the prediction of the secondary structure of hTom40A and AfTom40. Sequence conservation

and UV-CD spectra data were incorporated in the secondary structure prediction. The oligomerization index Q^3 for the presumably 19-stranded β -barrel indicated the presence of higher-order protein complexes. We examined the predicted lipid facing residues and those with relatively higher energy values are predicted to be weakly stable. The calculation of energy values of individual β -strand residues were performed as described in reference ³. Briefly, we calculated the energy of each residue in the predicted native conformation using an empirical potential function TmSIP derived from bioinformatics analysis of β -barrel membrane proteins ³⁶, first developed in ^{20; 37; 38}, and further refined with additional structural data in ^{3; 4}. The energy for each residue consists of two terms. First, each residue is assigned an energy value of burying this residue type at a particular depth in the lipid bilayer and with the orientation of its side-chain. There are two possible orientations, namely, side-chains facing the lipid environment or facing inside the barrel. This is termed the “single body term”. Second, each residue interacts with two residues on separate neighboring strands through strong backbone H-bond interaction, side-chain interactions and weak H-bond interactions, which collectively make up the two-body energy term. Strand energy is the summation of both single body and two-body energy terms over all residues in the strand.

Cloning and strains

For recombinant expression of wild-type hTom40A in *E. coli*, a truncated version of the protein lacking amino acid residues 1 to 82 was used (Fig. 1A). The corresponding gene (*hTom40A* 1-82) was PCR-amplified and cloned into a pET24d vector (Novagen) introducing a C-terminal His6-tag into the protein for purification {Mager, 2011 #1146}. The triple mutant *hTom40A* 1-82^{mut} (K107L, H117L and H220L) was prepared by site-directed mutagenesis using *hTom40A* 1-82 as a template and Pfu Ultra II DNA Polymerase (Agilent Technologies Inc.). First, replacement of amino acids K107L, H117L was accomplished by using primers for-K107L-H117L and rev-K107L-H117L (Table 1). Second, to generate the mutation H220L the plasmid containing mutations K107L and H117L was further mutagenized with primers for-H220L and rev-H220L (supplementary Table 1). Single hTom40A mutants K107L and H117L were made by site directed mutagenesis of *hTom40A* 1-82 by using the primers for-H117L/rev-H117L and for-K107L/rev-K107L, respectively (Table 1). In all cases, methylated parental plasmids were specifically digested with DpnI (New England Biolabs). Final plasmids were verified by DNA sequencing. Eventually, all vectors were transformed into *E. coli* BL21-CodonPlus (DE3) (Stratagene, Agilent Technologies) cells for protein expression.

The protein sequence of Tom40 from *Aspergillus fumigatus* was retrieved from the *Aspergillus* Genome Database. The corresponding gene and an *AfTom40* gene coding for a protein with mutations K69H, N150H, S180L, S236A and K302H were synthesized and cloned into a pET24d vector (GeneArt / Invitrogen). Both genes encoded for proteins with a carboxy-terminal hexahistidine tag. To produce these *AfTom40* proteins, vectors containing wild-type and mutant *AfTom40* were transformed into *E. coli* C41(DE3) (Lucigen) and BL21(DE3) (Stratagene, Agilent Technologies) cells, respectively.

Protein expression and isolation of inclusion bodies

Wild-type human Tom40A (hTom40A 1-82) and mutated human Tom40A (hTom40A 1-82^{mut}) were expressed forming inclusion bodies in *E. coli* BL21-CodonPlus (DE3) under tight control of expression from the T7 promoter. To obtain wild-type protein, BL21-CodonPlus (DE3) cells were transformed with pET24d-*hTom40A* 1-82 and grown in LB medium (20 ml) containing kanamycin (50 μ g/ml) at 37 °C in shaking flasks. This culture was used to inoculate LB medium (2 l) containing kanamycin (50 μ g/ml) for high level protein expression. Protein expression was induced with 1 mM isopropyl- β -D-

thiogalactopyranoside (IPTG) at a cell density corresponding to an OD₆₀₀ of 0.6. After 6 h of growth, cells were harvested by centrifugation, washed with phosphate-buffered saline and stored at -20 °C until further use. Mutant hToma40A protein (hTom40A 1-82^{mut}) was expressed by the same procedure. For purification of wild-type and mutant Tom40, cells were thawed and 3 ml lysis buffer (50 mM Tris-HCl pH 8, 1 mM EDTA, 100 mM NaCl, 1 mM phenyl methyl sulfonyl fluoride, 0.26 mg/ml lysozyme) were added per g cells. After lysis of membranes with 4 mg deoxycholate per g cells, DNaseI (12.5 units/g cells, Sigma-Aldrich) from bovine pancreas was added^{40; 41}. After incubation on ice for 20 min, inclusion bodies were separated from cell debris by centrifugation at 20000 × g for 30 min at 4 °C. Inclusion body pellets were washed with buffer containing 50 mM Tris-HCl and 100 mM NaCl, pH 8 and subsequently solubilized in 6 M guanidine hydrochloride (GnHCl), 20 mM Tris-HCl, 150 mM NaCl, 1 mM -mercaptoethanol, pH 8, using a glass-glass homogenizer. To remove insoluble material the homogenate was centrifuged at 30000 × g for 30 min at 4 °C and supernatants were stored at 4 °C. One liter cell culture yielded 0.5 - 1 g of unprocessed protein. Protein concentrations were determined by UV absorbance using an extinction coefficient of ϵ_{280} of 29900 M⁻¹cm⁻¹ for hTom40A 1-82 and hTom40A 1-82^{mut}³⁹.

For the over-expression of wild-type and mutant AfTom40, protein production was performed as for hTom40A with the difference that after induction the cells were further grown at 37 °C for 19 hours and cells were harvested by centrifugation. Cell pellet was stored at -20 °C until further use (~4 g/l of cell culture). The isolation of inclusion bodies was conducted under the same conditions for both AfTom40 wild-type and mutant and was based on the deoxycholic acid method according to^{40; 41}. Cells were thawed on ice and resuspended in 30 ml of lysis buffer (50 mM Tris-HCl pH 8.5, 1 mM EDTA, 100 mM NaCl) for 10 gram of cells. After the addition of phenyl methyl sulfonyl fluoride and lysozyme to a final concentration of 0.13 mM and 0.26 mg/ml, respectively, solution was incubated on ice for 20 minutes and stirred occasionally. Then, solution was transferred into a water bath at a temperature of 37 °C. In the following, 40 mg of deoxycholic acid was added and solution was stirred until it became viscous. 250 units of Benzonase® (Novagen) were added at room temperature and suspension was stirred until it was no longer highly viscous. Solution was further spun down at 19.600 × g for 30 minutes at 4 °C. Supernatant was discarded and pellet was resuspended in 100 ml of TNBP buffer (50 mM Tris-HCl pH 8.5, 100 mM NaCl, 1 mM -mercaptoethanol, 1 mM PMSF). After homogenization using a glass homogenizer, a clarifying spin was applied at 20.000 × g for 30 minutes at 4 °C. Retrieved inclusion body pellet was resuspended in 95 ml of TNTBP buffer (TNBP buffer containing 0.1 % (v/v) triton X-100) and homogenized on ice. A second clarifying spin was applied under the same conditions. Eventually, the pellet was washed with 100 ml of TNBP buffer and IB pellet was retrieved after centrifugation under the above named conditions. Washed IB pellet was then stored at -20 °C until further use. Determination of protein concentration was conducted as for human Tom40 by using extinction coefficients³⁹ of ϵ_{280} of 37025 M⁻¹cm⁻¹ for wild-type and mutant AfTom40, respectively.

Protein purification and folding

Inclusion bodies containing wild-type and mutated hTom40A (hTom40A 1-82 and hTom40A 1-82^{mut}) were loaded onto an Ni-Sepharose HiTrap column (1 – 20 ml, GE Healthcare) equilibrated with 6 M guanidine hydrochloride, 20 mM Tris-HCl, 150 mM NaCl, 1 mM -mercaptoethanol, pH 8. After washing the column with 2 column volumes of equilibration buffer, unspecifically bound proteins were removed with 20 mM imidazole. Then, hTom40A proteins were eluted with 300 mM imidazole, and fractions containing Tom40 were merged. Protein concentrations were adjusted to 5 mg/ml and samples were stored at 4 °C.

For refolding of wild-type and mutant hTom40A, purified protein in guanidine hydrochloride was diluted tenfold into 20 mM Tris-HCl pH 8, containing 0.5 % lauryldimethylamine-oxide (LDAO) and 1 mM β -mercaptoethanol. After removal of aggregates by centrifugation at $100000 \times g$, samples containing refolded protein were concentrated to ~ 5 mg/ml by Ni-NTA affinity chromatography. Samples were loaded onto Ni-Sepharose HiTrap columns (1 – 20 ml) previously equilibrated with 20 mM Tris-HCl pH 8, 0.1 % LDAO and 1 mM β -mercaptoethanol. Bound protein was eluted in the same buffer containing 300 mM imidazole. Final purification of protein was achieved by size exclusion chromatography using a Superose 12 column (GE Healthcare) which had been preequilibrated with 20 mM Tris-HCl pH 8, 0.1 % LDAO and 1 mM β -mercaptoethanol. The purity of isolated protein was assessed by SDS-PAGE followed by Coomassie Brilliant Blue staining, silver staining or Western blotting using antibodies against human Tom40 (Santa Cruz Biotechnology).

For refolding of wild-type and mutant Aftom40A the inclusion body pellets with both proteins were resuspended and homogenized in 6 ml of binding buffer (50 mM Tris-HCl pH 7.5, 8 M urea, 1 mM β -mercaptoethanol) per gram cells. Solution was centrifuged at $19.600 \times g$ and supernatant was loaded onto a Ni-Sepharose HiTrap column (1 – 5 ml, GE Healthcare) at room temperature. After column was washed with 10-15 column volumes of binding buffer, a gradient of 0-1 M imidazole was applied. Fractions containing Aftom40 were adjusted to a protein concentration of 6 mg/ml; and 6 mg of protein were refolded in 10 ml of refolding solution (20 mM NaH_2PO_4 pH 7, 1 mM β -mercaptoethanol and 0.8 % (w/v) polyoxyethylene monolauryl ether (Brij35)). The refolded protein solution was dialyzed at 4 °C against 50-100 fold volumetric excess of refolding buffer using a 6-8 kDa MWCO Filter (Spectra/Por®). Purity was then determined throughout SDS-PAGE and Coomassie Brilliant Blue. If necessary, a second purification step was conducted using the same conditions with the variation of exchanging 8 M urea against 0.8 % (w/v) Brij35 in the binding and elution buffer. Purified protein was then further dialyzed against 50-100 fold volumetric excess of refolding buffer and protein concentration was then adjusted to 0.2-0.4 mg/ml as needed.

Chemical cross-linking

For cross-linking experiments 40 μg of refolded and purified hTom40A 1-82 and hTom40A 1-82^{mut} were suspended in 100 μl 20 mM NaH_2PO_4 ; pH 8 and incubated with 125 μM freshly prepared glutaraldehyde at 37 °C for 0-45 min. Aliquots were removed and cross-linking reactions were stopped by adding Tris pH 8 to a final concentration of 50 mM. Finally, cross-linking products were analyzed by SDS-PAGE and Western blotting. Polyclonal antibodies against hTom40A were obtained from Santa Cruz Biotechnology, Heidelberg.

Circular dichroism

Circular dichroism (UV-CD) spectroscopy measurements of refolded and purified wild-type and mutant hTom40A (~ 0.2 mg/ml) in 20 mM Tris-HCl pH 8, 0.5 % (w/v) LDAO, 1 mM β -mercaptoethanol were performed using a Jasco J-715/815 spectrometer (Tokyo, Japan) in quartz cuvettes of 0.1 cm path length out of three independent protein preparations. Spectra were recorded at 25 °C from 185 to 260 nm with a resolution of 1.0 nm and an acquisition time of 20-100 nm/minute. Final CD spectrum was obtained by averaging five consecutive scans and corrected for background by subtraction of spectrum of protein-free samples recorded under the same conditions. Melting curves were recorded at constant wavelength at 216 nm for hTom40-A(1-82) wild-type (wt) and mutant hTom40A from 25 to a maximum of 98 °C by applying a temperature ramp of 1 °C/minute. All CD samples were filtered (Rotilabo® filter, pore size 0.22 μm) and spun down at full speed with a bench top centrifuge for five minutes at room temperature (Biofuge fresco, Heraeus; Newport Pagnell)

before measurements were conducted. Temperature readings displayed an error of $\sim 1^\circ\text{C}$, which was added to the experimental error. The mean residue ellipticity $[\theta]$ was calculated based on the molar protein concentration and the number of amino acid residues of regarding Tom40 proteins. Secondary structure content was determined using the CDpro package, namely CDSSTR, CONTIN/LL and SELCON 3^{42; 43; 44}.

AfTom40 proteins were measured under the same conditions at 0.1–0.4 mg/ml 20 mM NaH_2PO_4 pH 7, 1 mM β -mercaptoethanol and 0.8 % (w/v) Brij35.

To fit the unfolding curves of mutant Tom40s we assumed a two-state unfolding mechanism. The fraction $f_U(T)$ of unfolded protein was calculated according to $f_U(T) = ([\theta] - [\theta]_N) / ([\theta]_U - [\theta]_N)$ and fitted to the sigmoid function $f_U(T) = 1 / [1 + e^{(A/T - B)}]$. $[\theta]_N$ and $[\theta]_U$ represent the ellipticities of the native and unfolded molecule. At low and high temperatures, $[\theta]_N$ and $[\theta]_U$ increase linearly with temperature according to $[\theta]_N = aT + b$ and $[\theta]_U = cT + d$. A and B are fitting parameters. If unfolding is fully reversible, they correlate with enthalpic and entropic changes of the unfolding reaction, respectively^{45; 46; 47}. Wild type Tom40 curves were fitted by superposition of two sigmoid functions assuming a three-state unfolding mechanism⁴⁸.

Tryptophan fluorescence measurements

Tryptophan fluorescence spectra of wild-type and mutant hTom40A (~ 0.15 mg/ml) were recorded by a FP-6500 spectrofluorimeter (Jasco Inc.) after 24 h of incubation in 20 mM Tris-HCl, pH 8, 1 % (w/v) LDAO, 1 mM β -mercaptoethanol, and 0.35 to 7.0 M guanidine hydrochloride at 25°C . Tryptophans were excited at 280 nm. Emission spectra were recorded between 300 and 400 nm with an integration time of 1 s. The band width for excitation and emission was set to 3 nm, respectively. Fluorescence spectra were evaluated by fitting background-corrected spectra $I(\lambda)$ to the log-normal distribution $I(\lambda) = I_0 e^{-[\ln 2 / \ln 2] \cdot \ln^2[1 + ((\lambda - \lambda_{\text{max}}) / \Delta\lambda)^2]}$ ^{49; 50}, where I_0 is the fluorescence intensity observed at the wavelength of maximum intensity λ_{max} , $\Delta\lambda$ is the line shape asymmetry parameter and $\Delta\lambda$ is the spectral width at half-maximum fluorescence intensity $I_0/2$. Wild-type and mutant AfTom40 (~ 0.1 mg/ml) were investigated using the same parameters in 20 mM NaH_2PO_4 pH 7, 1 mM β -mercaptoethanol and 0.8 % (w/v) Brij35.

To characterize denaturant induced folding and unfolding of mutant hTom40A and AfTom40 the fraction of unfolded protein $f_U(D)$ was fitted by the sigmoid function $f_U(D) = 1 / [1 + e^{(A - B[D]) / T}]$. If unfolding and folding are fully reversible, A correlates with the free energy of the protein that describes its stability at zero denaturant concentration. In this case B would be a measure of the dependence of free energy on denaturant concentration $[D]$ ^{22; 23; 24}. Chemically induced unfolding of wild-type Tom40s was processed as described for CD measurements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Expected energy levels of strands and residues in the transmembrane domain of hTom40A. (A) Secondary structure prediction of hTom40A is based on PRED-TMBB³⁴ and TMBETAPRED-RBF³⁵. (B) Protein topology of hTom40A was generated using TMRPres2D⁵¹. (C) Empirical energy of α -strands 1 - 19 of wild-type protein. α -strands 1, 2 and 9 are predicted as the weakly stable strands. (D) Single body propensities of amino acid residues of the transmembrane domains of α -strands 1, 2 and 9. Amino acids K107, H117, and H220 show the highest values indicating a destabilizing effect on the regarding strand. (E) Empirical energy of α -strands 1 - 19 of mutant (K107L, H117L and H220L) human Tom40A.

Fig. 2.

Far-UV CD spectra and tryptophan fluorescence emission spectra of wild-type and mutant hTom40A. (A) SDS-PAGE showing purified wild-type (wt) and mutant (K107L; H117L; H220L; K107L/H117L; K107L/H117L/H220L) human Tom40A. Proteins were visualized by silver staining. (B) Comparison of far-UV CD spectra between wild-type and mutant hTom40A. Measurements were carried out at a protein concentration of ~0.2 mg/ml at 25 °C for wild-type and mutant proteins, respectively. For each experiment, five scans were accumulated at the indicated temperatures. Noisy data below 200 nm due to optical density has been removed. (C) Comparison of tryptophan fluorescence emission spectra of wild-type and mutant hTom40A. Emission spectra were conducted in 20 mM Tris-HCl, pH 8, 1 % (w/v) LDAO, 1 mM β -mercaptoethanol and 350 mM guanidinium hydrochloride at a protein concentration of ~0.15 mg/ml for all isoforms. Data points were fitted to the log-normal distribution as described in materials and methods. Relative fluorescence intensity is in arbitrary units (a.u.). All isoforms exhibit the exact same far UV-CD and tryptophan fluorescence emission spectra characteristics. We suggest analogue β -barrel formation for wild type and mutant hTom40A.

Fig. 3.

Sensitivity of wild-type and mutant hTom40A to thermal denaturation. Thermal denaturation of both hTom40A isoforms was monitored under the same conditions as described in figure 2B by change of ellipticity at 216 nm. Wild-type and mutant protein were subjected to temperature increases of 1 °C/min from 25 to 98 °C, respectively. Data points of melting curves were normalized to the minimum and maximum percentage of unfolded protein fraction ($n = 3$)⁵² and then fitted to sigmoid functions (black line). Apparent melting temperatures were retrieved at the midpoint of the transition curves. Wild-type Tom40A indicated a three-state unfolding mechanism, whereas the triple mutant hTom40A showed a two-state unfolding mechanism with an approximately 11 °C higher apparent melting temperature.

Fig. 4.

Sensitivity of wild-type and mutant hTom40A to chemical denaturation. (A and B) Tryptophan fluorescence emission spectra of wild-type and mutant hTom40A ~0.15 mg/ml were recorded in Tris-HCl buffer containing 1 % LDAO after 24 h incubation with guanidinium hydrochloride (GnHCl) at 25 °C. GnHCl concentrations varied from 0.35 to 7 M, respectively. (C) The fractions of unfolded wild-type and mutant Tom40 were determined at different GnHCl concentrations from fluorescence intensities recorded at 330 nm. (C and D) The fractions of unfolded wild-type and mutant Tom40 were determined at different GnHCl concentrations from fluorescence intensities recorded at 330 nm relative to a denaturated (C) and a renaturated (D) protein solution. To allow direct comparison of the reversibility of the unfolding reaction, the fractions of unfolded wild-type and K107L/H117L/H220L hTom40A shown in panel (C) are included also in (D) and fitted with solid lines. The figures show an average (sem $\pm$ sd) of three independent experiments.

Fig. 5. Stability analysis of Tom40 from *Aspergillus fumigatus*. (A) Empirical energy of α -strands 1 - 19 of wild-type and mutant protein. (B) SDS-PAGE of purified and refolded wild-type (wt) and mutant (K69H, N150H, S180L, S236A and K302H) AfTom40 throughout Coomassie blue staining. Far-UV CD spectra (C) and tryptophan fluorescence emission spectra (D) of AfTom40wt/mutant were recorded and deconvoluted as described in Fig. 2, respectively. Both AfTom40 isoforms adapt the same secondary structure (C), but mutant AfTom40 showed an approx. 1.5 nm left-shifted tryptophan fluorescence emission spectrum. Thermal (E) and chemical (F) sensitivity of AfTom40 isoforms was investigated according to Fig. 3 and 4C. Thermal and GnHCl induced unfolding of proteins revealed a three-state unfolding mechanism for the wild-type and a two-state unfolding mechanism for the mutant protein with a higher stability for the mutated form of AfTom40.

Fig. 6. Chemical cross-linking of wild-type and mutant (K107L, H117L and H220L) hTom40A. Wild-type and mutant protein (40 μ M) were incubated with glutaraldehyde (125 μ M) at 37 $^{\circ}$ C in 20 mM NaH_2PO_4 ; pH 8. Aliquots were taken after the addition of the cross-linking reagent at the time indicated. In these, cross-linking reactions were stopped by addition of 50 mM Tris, pH 8. Proteins and cross-linking products were visualized by SDS-PAGE and Western blotting.