Structure of the cytosolic domain of TOM5, a mitochondrial import protein

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Abstract TOM5 is a small outer mitochondrial membrane protein in *Saccharomyces cerevisiae* and is part of a multi-protein translocator complex, which mediates protein import into mitochondria. Presently, nothing is known about the conformational preferences of TOM5 or other mitochondrial import proteins. In this report, circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy are used to determine the conformational preferences of the cytosolic domain of TOM5. The CD spectra show evidence of a helical structure that is invariant with pH. NOESY data revealed that TOM5 forms a stable helical core between E11 and R15 with a less structurally rigid helix extending to the C-terminus.

Key words: TOM5; Mitochondrial protein import; Nuclear magnetic resonance; Conformation

1. Introduction

TOM5 is a 50 residue protein of the outer mitochondrial membrane in *Saccharomyces cerevisiae* and is part of a multi-protein complex which mediates protein import into mitochondria [1]. Despite its small size, TOM5 is an integral membrane protein that appears to be oriented with its N-terminal portion exposed to the cytosol. After membrane disruption, TOM5 was found with a complex that included TOM40 and TOM22. In addition, TOM5 could be cross-linked to pre-proteins, indicating its proximity to them. The TOM5 sequence includes six glutamic acids. This acidic nature led to the inclusion of TOM5 in a hypothetical 'acid chain' [2], in which pre-proteins are transferred among a series of membrane proteins that have a progressively increasing affinity for naturally basic N-terminal presequences [3]. The cross-linking data and acidity suggest that TOM5 could interact with presequences during import. Presequences tend to form positively charged amphiphilic α-helices when induced by lipid surfaces or structure promoting organic co-solvents [4–7], but little detailed structural information is available on their interaction with TOM proteins. In this paper, we report the conformational properties of the cytosolic TOM5 segment and attempt to observe its interaction with presequence peptides.

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2. Materials and methods

2.1. Peptide synthesis and purification

TOM5[1–26] was synthesized using an ABI 430A peptide synthesizer in the Laboratory for Macromolecular Structure in the Purdue University Department of Biochemistry. To protect against the interference of secondary structure formation during peptide synthesis, tBOC protecting groups were used. The crude peptide was purified using a semi-preparative C18 reverse-phase high performance liquid chromatography column (Vydac) and applying an acetonitrile gradient. The solution was frozen on dry ice and lyophilized. The identity of the resulting white powder was confirmed by mass spectrometry and amino acid analysis.

2.2. Circular dichroism (CD) spectroscopy

CD spectra were obtained using a Jasco J600 spectropolarimeter and a cell with a 0.1 cm path length. Sample concentrations were in the range of 5–10 μM and temperature was maintained with the use of an external circulating bath. Spectral data were loaded into the program Origin (Microcal Software, Northampton, MA, USA) for preparation of spectral figures.

2.3. Nuclear magnetic resonance (NMR) spectrometry

NMR spectra were obtained using a Varian Unity+600 MHz instrument. The peptides were dissolved in 50 mM phosphate, 50 μM EDTA, 50 μM azide, pH 7.2, to a final concentration of ca. 1.5 mM. One sample contained 10 volume% trifluoroethanol (TFE). Spectra were obtained at both 10° and 15°C. Clean-TOCSY spectra [8] utilized 30 ms and 70 ms mixing periods, while mixing times for NOESY spectra were 200 ms. Two-dimensional transformations were performed [9] using NT NMRPipe software [10] on a Dell 400-series workstation running Microsoft Windows NT.

2.4. Fluorescence spectroscopy

Fluorescence spectra were obtained with the use of a Hitachi instrument. The peptides were dissolved in 50 mM phosphate, 50 μM EDTA, 50 μM azide, pH 7.2, to a final concentration of ca. 5 μM in a total volume of 1.5 ml. Aliquots of TOM5[1–26] were added until a final concentration of 10 μM was achieved. Changes in tryptophan fluorescence were monitored after excitation at 290 nm. Emission was scanned from 310 to 400 nm at a temperature of 25°C.

3. Results

Analysis of the TOM5 amino acid sequence suggested that residues 1–26 comprise the cytosolic domain of the protein, while residues 27–45 formed a membrane-spanning segment [1]. A peptide corresponding to residues 1–26 was synthesized, purified and found to be readily soluble in phosphate buffer at pH 3.0, 5.9 and 7.2. CD spectra were obtained as a function of temperature and pH. All spectra showed the presence of ordered secondary structure. The spectra obtained at 25°C at each pH are shown in Fig. 1. Secondary structure estimation using the CD intensity at 222 nm [11] indicated that at each pH, the peptide was approximately 10% helical. At 10°C, the helical content increased to about 20% and did not vary significantly with pH, even at temperatures as high as 50°C.

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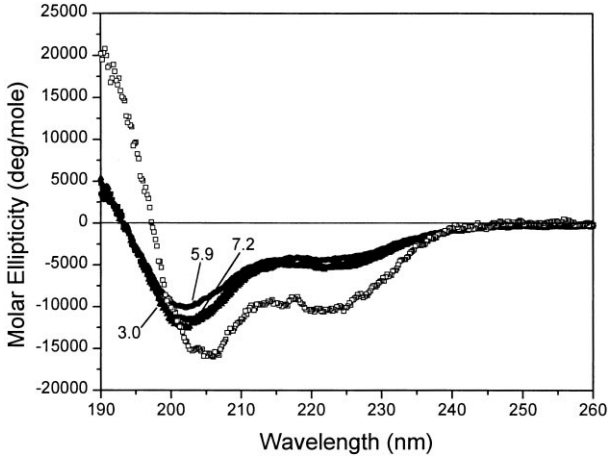

Fig. 1. Far UV CD spectra of TOM5[1–26] in phosphate buffer, pH 7.2 (■), 5.9 (●) and 3.0 (▲). Molar ellipticity ([Θ]) is in units of °/mol. Also shown is the spectrum at pH 7.2 with 10% (v/v) TFE (□). All spectra were obtained at 25°C with 10 μM sample concentrations. Eight scans were accumulated for each spectrum.

Invariant CD spectra with changing pH suggest that helix formation and stability were not aided by the formation of ionic interactions among the side-chains that occurred with helical periodicity. When the CD spectrum was measured in the presence of 10% TFE, the helical content increased (Fig. 1). From the comparison of spectra with and without TFE, it cannot be determined whether the helical segment became longer or, for a segment of constant length, the helix/coil equilibrium was shifted toward the helical conformation.

To obtain sequence-dependent structural information, TOM5[1–26] was analyzed by NMR spectroscopy in 50 mM phosphate, pH 7.2. Proton resonance assignments were obtained from TOCSY experiments (30 and 70 ms) at 10° and 15°C and NOESY experiments were used to establish sequential resonance assignments. Complete chemical shift assignments are shown in Table 1. Secondary structure was assessed from inter-residue interactions observed in the NOESY spectra. Although cross-peak intensities were low, consistent evidence for α-helical structure began with S10 and continued through Q20 (Fig. 2A). A secondary structure analysis based on δ^1Hα values [12] identified E11–R15 as the only region that could be characterized as helical (Fig. 2C). When the buffer solution contained 10% TFE, the helical character of the peptide increased significantly, a result consistent with the CD data. In 10% TFE, helix-dependent NOESY cross-peaks became more intense between E11 and Q20 (Fig. 2B) and extended farther toward the C-terminus. The observed δ^1Hα values corresponded to the helical character from E11 through L25 (Fig. 2C). Both NOESY and chemical shift data reveal only small differences among the eight residues at the N-terminus.

As stated previously, the CD data corresponded to 10% helix at 25°C and 20% helix at 10°C. The segment E11–R15 comprises 20% of the peptide sequence. If the A16–L25 segment made only a minor contribution to the observed helical character, at 25°C, E11–R15 was ca. 50% helical and at 10°C, it was nearly 100% helical. These percentages represent the upper limits of helicity for E11–R15. The helical nature of the polypeptide was enhanced in 10% TFE with a consistent helical pattern from E11–L25. The most striking chemical shift differences were observed in residues at the N-terminal end of the helical segment (V9, S10, E11 and E12). It was probably the increase in helicity and the helical dipole that led to these chemical shift changes. The N-terminal segment was essentially unaltered by the presence of TFE.

Several spectroscopic methods were used in attempts to

Table 1
Sequential resonance assignments of TOM5[1–26] in phosphate buffer at 10°C

Residue	NH	Hα	Hβ	Hγ	Hδ	Others
M1	–	4.07	2.09, 2.09	2.51, 2.51		
F2	8.86	4.66	3.17, 3.12		7.29	7.33, 7.39
G3	8.42	3.91, 3.83				
L4	8.14	4.63	1.58, 1.61	1.67	0.94, 0.94	
P5	–	4.42	2.34, 2.06	2.06, 1.90	3.88, 3.66	
Q6	8.61	4.24	2.08, 2.03	2.40, 2.40		
Q7	8.55	4.30	2.09, 2.03	2.39, 2.39		
E8	8.45	4.35	2.09, 2.01	2.42, 2.40		
V9	8.25	4.08	2.09	0.96, 0.96		
S10	8.47	4.43	4.11, 3.98			
E11	8.66	4.17	2.11, 2.11	2.45, 2.45		
E12	8.48	4.11	2.09, 2.09	2.45, 2.45		
E13	8.18	4.16	2.14, 2.08	2.46, 2.38		
K14	8.20	4.12	1.89, 1.89	1.68, 1.68	1.56, 1.39	2.96, 2.96
R15	8.14	4.20	1.89, 1.89	1.75, 1.65	3.23, 3.23	7.38
A16	8.14	4.21	1.41			
H17	8.31	4.62	3.36, 3.27			8.63, 7.33
Q18	8.31	4.25	2.15, 2.13	2.44, 2.41		
E19	8.51	4.25	2.09, 2.07	2.49, 2.41		
Q20	8.36	4.32	2.15, 2.10	2.44, 2.44		
T21	8.21	4.25	4.25	1.21		
E22	8.37	4.29	2.10, 2.05	2.46, 2.42		
K23	8.28	4.27	1.86, 1.79	1.69, 1.69	1.49, 1.43	3.01, 3.01
T24	8.15	4.28	4.24	1.23		
L25	8.29	4.35	1.70, 1.70	1.61	0.93, 0.88	
K26	8.31	4.31	1.88, 1.83	1.71, 1.71	1.51, 1.44	3.01, 3.01

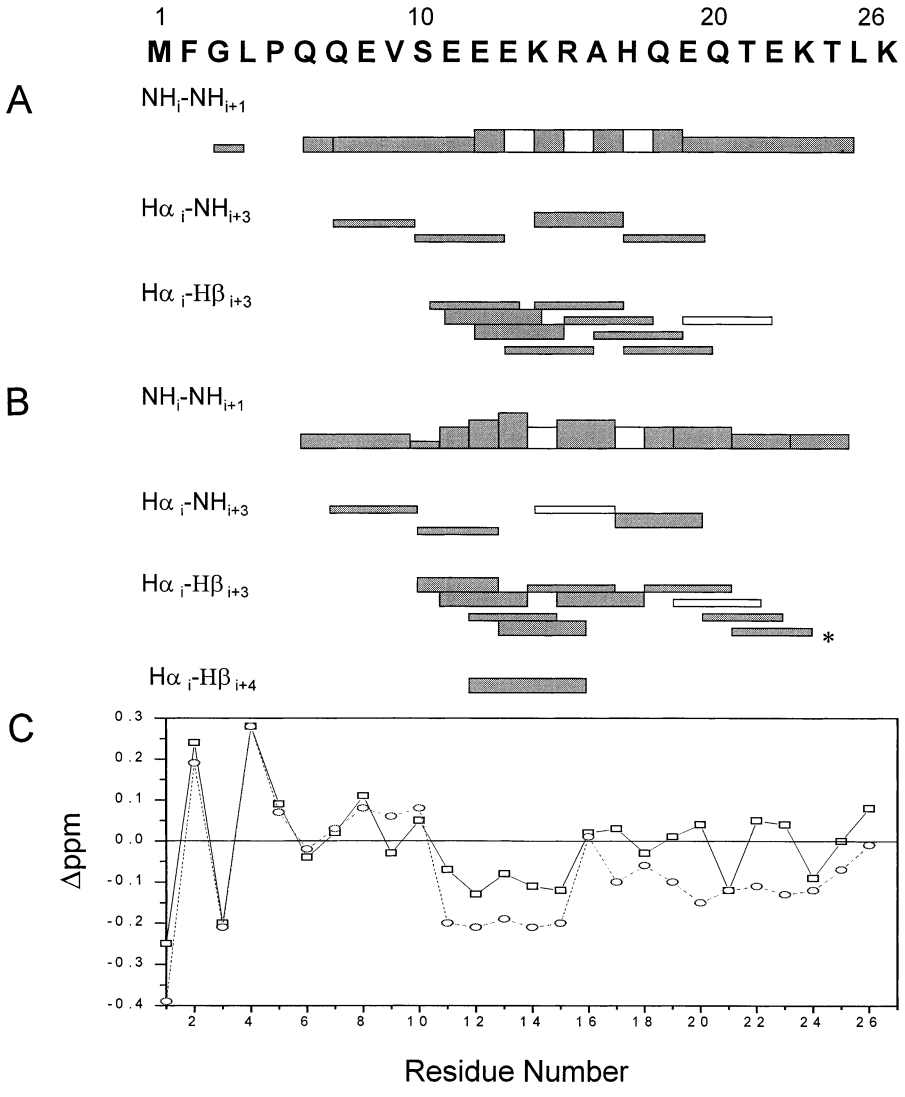

Fig. 2. NMR analyses of TOM5[1–26] conformation. Inter-residue NOEs from NOESY spectra ($\tau_m = 200$ ms) observed for TOM5[1–26] in (A) phosphate buffer, pH 7.2, and (B) phosphate buffer, pH 7.2, with 10% (v/v) TFE. Bar thickness denotes relative cross-peak intensity. The asterisk (*) indicates a $H\alpha_i$-$H\gamma_{i+3}$ interaction. (C) Chemical shift differences (Δppm) between observed δ^1Hα values in buffer (□) and 10% TFE (○) and standard random coil values [12] plotted versus the residue number.

observe interactions between TOM5[1–26] and presequence peptides. The rhodanese sequence, including W15, and the L2W mutant of the ALDH presequence [13] were utilized to monitor fluorescence changes when the peptides were combined with TOM5[1–26]. A series of aliquots containing the targeting peptides were added to a cuvette containing buffer alone and to TOM5[1–26] in buffer. No differences were observed in Trp fluorescence of either rhodanese or L2W-pALDH. Similarly, only non-significant differences were observed in the CD spectra of TOM5[1–26] when the peptide was combined with equi-molar amounts of either rhodanese or ALDH peptide in a CD cell in both buffer and 10% TFE.

4. Discussion

We have presented previously unknown information regarding the conformational preferences of an outer mitochondrial membrane protein involved in protein import. Unlike many other peptides of similar size, TOM5[1–26] displays a helical character when dissolved in aqueous buffer. NMR data showed the helical region to extend from E11–K26. While the peptide displayed a natural tendency toward helix formation,

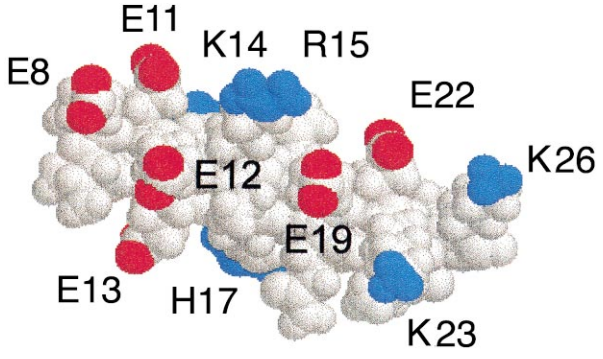

Fig. 3. Space-filling model of helical TOM5[1–26] beginning with E8 and showing alternating negatively charged (red) and positively charged (blue) patches.

the helical character was enhanced by the addition of a small amount of TFE. Properties of residues M1–E8 in TOM5-[1–26] were indifferent to the addition of TFE, making it appear rather difficult to induce this segment to form a helix. The C-terminal portion of TOM5 consists of a series of non-polar residues (27–45) and a short C-terminal tail (46–50). The length of the non-polar segment is sufficient to span the membrane as an α-helix. The helical region from E11 to R15 and a helical transmembrane segment ought to have a stabilizing effect on the secondary structure of the intervening residues, as did TFE. The data lead to a model of TOM5, in which a relatively disordered 10 residue segment at the N-terminus is followed by a 35 residue helix with E11–K26 in the cytosol and Q27–V45 in the membrane. The helical cytosolic region of TOM5 displays a topology that features alternating regions of negative and positive charge (Fig. 3). The alternating charge distribution does not present a complementary charge pattern to typical N-terminal mitochondrial presequences. In addition, there is no hydrophobic surface that would interact favorably with the hydrophobic portion of a presequence.

At the present time, we have been unable to directly observe interactions between TOM5[1–26] and the targeting peptide of rhodanese or ALDH. It is possible that an undetected interaction occurred. The CD experiments were designed to detect secondary structure changes upon interaction between TOM5[1–26] and the targeting peptides. Fluorescence measurements were based on changes in the environment of tryptophan side-chains. If, despite binding, neither of these events occurred, it would appear that no interactions had taken place. It is also possible that the transmembrane segment of TOM5 could alter the structure of residues 1–26, enabling the interaction with targeting sequences. However, according to the model we have proposed, it is unlikely that TOM5 interacts directly with the typical positively charged and amphiphilic targeting sequence. Neither the charge distribution nor the hydrophobicity of TOM5 is conducive to this type of interaction.

When the TOM5 gene was deleted from yeast, import of proteins was reduced, regardless of their intra-mitochondrial location [1]. It was subsequently suggested that, rather than participating in the 'acid-chain', TOM5 may function as an assembly component of the translocation complex [14]. More recently, it was shown that the import of small TIM proteins is dependent on the presence of TOM5 and not the larger TOM import receptors [15]. The N-terminal segments of these TIM proteins are not positively charged, so they have either internal targeting information or they may be able to interact preferentially with the cytosolic domain of TOM5. While our binding results are inconclusive, they are consistent with these other published data.

This report constitutes the first detailed structural data among TOM proteins. We cannot draw specific conclusions about the function of TOM5 from this structure alone because it appears that interactions with other proteins must be considered. Regardless, the helical structure and alternating charge topology of TOM5 should ultimately become part of a more thorough understanding of mitochondrial protein import.

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