A Biophysical Analysis of the Tetratricopeptide Repeat-rich Mitochondrial Import Receptor, Tom70, Reveals an Elongated Monomer That Is Inherently Flexible, Unstable, and Unfolds via a Multistate Pathway*

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Proteins destined for all sub mitochondrial compartments are translocated across the outer mitochondrial membrane by the TOM (translocase of the outer membrane) complex, which consists of a number of specialized receptor subunits that bind mitochondrial precursor proteins for delivery into the translocation channel. One receptor, Tom70, binds large, hydrophobic mitochondrial precursors. The current model of Tom70-mediated import involves multiple dimers of the receptor recognizing a single molecule of substrate. Here we show via a battery of biophysical and spectroscopic techniques that the cytosolic domain of Tom70 is an elongated monomer. Thermal and urea-induced denaturation revealed that the receptor, which unfolds via a multistate pathway, is a relatively unstable molecule undergoing major conformational change at physiological temperatures. The data suggest that the malleability of the monomeric Tom70 receptor is an important factor in mitochondrial import.

The majority of mitochondrial proteins are encoded on nuclear genes, synthesized via cytosolic ribosomes, and chaperoned to the outer membrane of the organelle, where it is recognized by receptors of the TOM complex (1–3). Upon binding, the precursor is transferred to the translocation pore of the complex, comprising the pore-forming Tom40 and its associated subunits Tom5, Tom6, and Tom7 (1–3). One of the two TIM (translocase of the inner membrane) complexes then completes the sorting of the imported protein into the appropriate compartment of the mitochondrion (1, 4).

Many mitochondrial precursor proteins are unusually hydrophobic (5), including proteins soluble in the matrix (such as the dehydrogenase Adh3), peripheral membrane proteins such as the β-subunit of the F1-ATPase, and integral membrane proteins such as members of the carrier family. These proteins have been shown to require the Tom70 receptor for efficient import into mitochondria (6–10). Many of these precursor proteins might make use of cytosolic chaperones (11), and recently, Tom70 was shown to act both as a receptor directly interacting with precursors and as a docking site for cytosolic forms of Hsp70. Tom70 is composed of multiple tetratricopeptide repeat (TPR) motifs: a degenerate 34-amino-acid sequence widely characterized as mediators of protein-protein interactions (12, 13). Structurally, a single TPR motif forms a pair of antiparallel α-helices that are often arranged in large super-helical arrays of between 3 and 20 repeats (14) that can form a contiguous surface suitable for binding to short segments of peptide ligand (15).

Sequence analysis of Tom70 from yeast and mammalian systems suggests that the receptor has seven TPR motifs (9, 16). The three N-terminal TPRs of Tom70 show a high degree of similarity to TPR motifs seen in Hsp70-binding proteins and perform a similar role in Tom70, serving as a clamp for the C-terminal peptide of Hsp70 (9, 17). By contrast, the TPR segments in the central region of Tom70 specifically recognize loops of the precursor ligand (18). A truncated form of Tom70, lacking the C-terminal segment, cannot suppress the temperature sensitivity of a Δtom70 mutant (19) and might represent a secondary ligand-binding site (16, 18). Precursor proteins are then transferred to the translocation channel, but this transfer depends on the interaction Tom70 makes with the single TPR segment in the Tom20 receptor (20).

The current model for how Tom70 assists the import of precursor proteins has the receptor existing as a homodimer that is only transiently associated with the TOM complex (9, 21, 22). Multiple dimers of Tom70 are thought to be required to bind each of the internal targeting sequences of the precursor and facilitate its transfer through the import pore of the TOM complex (9, 22). Evidence for the existence of Tom70 dimers stems from its apparent slow mobility under blue native gel electrophoresis (25) and the presence of a 500-kDa complex when ADP/ATP carrier-bound Tom70 is analyzed by the same technique (22). The transmembrane domain of Tom70 has been suggested to mediate oligomerization (23, 24); however, recent work indicates that these transmembrane domains may not associate in vivo (25). In this study, we present a biophysical characterization of the cytosolic domain of Tom70, the first such study involving a subunit of the mitochondrial import system.
apparatus. Tom70 is shown to be a monomer that adopts an elongated shape. This monomer is also shown to be intrinsically unstable, unfolding at physiological temperatures. We propose that the inherent fluidity of Tom70 is an important factor in mitochondrial import, allowing flexibility in the interactions it needs to make with Hsp70, precursor ligand, and Tom20.

MATERIALS AND METHODS

**Protein Expression and Purification**—DNA encoding residues 51–617 of Tom70 was amplified via PCR from *Saccharomyces cerevisiae* genomic DNA using the primers 5’-GTATTCATGGACACAAAGGATCTCAAAGG-3’ and 5’-AGAGGCCATGGATGTAACTGAAGCAG-3’. The resultant PCR product was digested with BspHI and BamHI restriction enzymes and cloned into the NcoI/BglII region of a pQE60 expression vector (Qiagen). Recombinant protein was expressed in a BL21(DE3) expression strain and purified via Ni²⁺/H⁺ affinity chromatography, anion exchange, and size exclusion chromatography. Protein was kept in a buffer comprising 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol. The protein concentration was determined by measuring absorbance at 280 nm.

**Spectroscopic Methods**—Fluorescence emission spectra were recorded on a PerkinElmer Life Sciences LS50B spectrofluorimeter using a temperature-controlled cuvette at 25 °C in a 1-cm path length quartz cell. Excitation and emission slits were set at 5.0 nm for all spectra, unless otherwise stated, and a scan speed of 10 nm/min was used. Circular dichroism spectra were measured on a Jasco 810 spectropolarimeter using a temperature-controlled cuvette at 25 °C. Far UV spectra from 190 to 250 nm were collected with 5-point signal averaging. Measurements were made with the signal averaged over 15 s. The concentration of the protein was 0.1 mg/ml with 0.1-cm path length. For a comparison of unfolding transitions collected at different protein concentrations, the data were normalized by calculating the fraction of unfolded protein as described previously (26). All unfolding data were fit to either a two- or a three-state unfolding model as described previously (27, 28).

**Analytical Ultracentrifugation**—A Beckman Optima model XL-A analytical Ultracentrifuge equipped with a photoelectric absorbance optical detection system was used for all sedimentation experiments. For sedimentation equilibrium experiments, 100 μl of Tom7051–617 (1.5 mg/ml) dissolved in 20 mM Tris-HCl, 50 mM NaCl, 1 mM dithiothreitol, pH 8.0, and 120 μl of reference were loaded into a conventional double sector filled epon centerpiece (path length 1.2 cm) with quartz windows and mounted in a Beckman An-60 Ti rotor. Data were collected at 280 nm with five averages, at time intervals of 240 min and radial intervals of 0.001 cm, until sedimentation equilibrium was attained (~16 h). The establishment of sedimentation equilibrium was ascertained by overlaying the data until no apparent movement was observed between consecutive data. Experiments were conducted at 20 °C with a rotor velocity of 15,000 rpm. To estimate the signal due to nonsettling contaminants, high speed depletion was performed at 40,000 rpm for at
least 3 h, and the resulting baseline offset ($E$) was calculated by averaging the absorbance over a radial range of 0.1 cm in the resulting plateau adjacent to the sample meniscus. For all samples, the values for $E$ represented <5% of the initial absorbance, indicating the presence of only minor amounts of low mass contaminants. Solvent densities and viscosities were computed using SEDNTERP (29). Molar extinction coefficients at 280 nm ($35,130M/\text{cm}^2$) and partial specific volume of Tom7051–617 (0.733 ml/g at 20 °C) were calculated from the amino acid composition. An equivalent molar mass ($M'$), assuming a single species, was obtained from the nonlinear least squares best-fit at a single speed according to the exponential relationship using the program SEDPHAT (30),

$$c(r) = c(r_0) \exp \left[ \frac{S}{v} - \frac{r^2}{r^2(r_0^2 - r^2)} \right] + E$$

(Eq. 1)

where $c(r)$ is the concentration at radius $r$, $c(r_0)$ is the concentration at the reference radius $r_0$, $v$ is the rotor angular velocity, $R$ is the gas constant, $T$ is the temperature, $\bar{v}$ is the partial specific volume of the Tom46450

**Biophysical Characterization of Tom70**

**FIG. 2.** Tom7051–617 is a monomeric protein. A, the elution profile of Tom7051–617 from a Superdex 200 (Amersham Biosciences) size exclusion column. Inset, standard curve of the S200 column calibrated with thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa). MW, molecular mass. B, sedimentation velocity profile of Tom7051–617 plotted as a function of absorbance at 230 nm versus the position from the axis of rotation at time intervals of 300 s. Solid lines represent nonlinear best-fit to a continuous sedimentation distribution model (28). C, continuous sedimentation coefficient, $c(s)$, distribution for Tom7051–617 at 0.25 mg/ml with a frictional ratio ($f/f_0$) of 1.7 and calculated with a resolution of 100 sedimentation coefficients between 0.5 S and 8.0 S employing maximum entropy regularization at a confidence level (P) of 0.95.
solvent, \( p \) is the solvent density, and \( E \) is the baseline offset.

For sedimentation velocity experiments, 380 \( \mu l \) of sample (0.25–2.0 mg/ml) and 400 \( \mu l \) of reference were employed under the same solution conditions as the sedimentation equilibrium experiments described above. Experiments were conducted at a rotor speed of 40,000 rpm, and the data were collected in continuous mode, at a single wavelength, a time interval of 300 s, and a step size of 0.003 cm without averaging. Multiple scans at different time points were fitted to a continuous size distribution model using the program SEDFIT (31).

RESULTS

Production of Recombinant Tom70\textsubscript{51–617}—The cytosolic domain of Tom70 (residues 51–617) was expressed in recombinant form and purified to homogeneity (Fig. 1A). Tom70\textsubscript{51–617} was shown to be functional, as it could bind \textit{in vitro}-translated phosphate carrier, a known substrate for Tom70 (18, 32, 33). The secondary structure of the Tom70\textsubscript{51–617} was analyzed by far UV circular dichroism (Fig. 1B). Tom70\textsubscript{51–617} was observed to have double minima at 222 and 208 nm, which is indicative of protein with a high helical content. Deconvolution of the CD data (using the method described by Aggerbeck et al. (34)) shows that Tom70\textsubscript{51–617} has an \( \alpha \)-helical content of 73%. Considering that the seven putative TPR motifs in Tom70 constitute 54% of the total structure of the protein, the predicted \( \alpha \)-helical content is in reasonable agreement with the observed data, indicating the presence of helical content in addition to that contained in the TPRs.

Recombinant Tom70\textsubscript{51–617} Is Monomeric—Tom70 has been suggested to form a dimer in the outer mitochondrial membrane with subunit association mediated by its transmembrane domain (23, 24). Previous studies had suggested Tom70\textsubscript{51–617} to be monomeric (18), whereas studies by Young et al. (9) with the same protein indicated that Tom70 elutes as a dimer via size exclusion chromatography. In our study, Tom70\textsubscript{51–617} eluted from size exclusion chromatography at a position equivalent to a 125-kDa globular protein, suggesting that Tom70\textsubscript{51–617} is dimeric (Fig. 2A). To investigate the solution properties of Tom70\textsubscript{51–617} further, the purified protein was subjected to sedimentation velocity analyses in the analytical ultracentrifuge. The absorbance \( r \) is plotted as a function of radial position (\( r \)) subtracted from the radius of the sample meniscus (\( r_m \)). The \textit{open circles} represent the sedimentation equilibrium data of Tom70\textsubscript{51–617} at an initial protein concentration of 0.5 mg/ml obtained at a rotor velocity of 15,000 rpm. The \textit{solid line} represents the nonlinear least squares best-fit to a single species according to Equation 1, yielding an equivalent molar mass of 56.7 kDa, which is consistent with the monomeric molar mass of the recombinant protein. Top panel, the residuals for the nonlinear best-fit described above are plotted as a function of radial position.

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To further characterize the structure of Tom70\(^{51–617}\)
(Fig. 2C), agreement with the monomeric mass of the protein (65.9 kDa) calculated to be 56.9 kDa (data not shown). This is in good agreement with the native state of Tom70\(^{51–617}\) at an initial concentration of 1.5 mg/ml was unfolded via a single transition exceeded 4 M. The lack of a concentration-dependent step in the unfolding pathway further suggests that the native state of Tom70\(^{51–617}\) is monomeric.

In addition, changes in the secondary structure of Tom70\(^{51–617}\) during unfolding were monitored by far-UV CD. The resultant data show that Tom70\(^{51–617}\) unfolds via a single intermediate (Fig. 6). From Equation 1, the equivalent molar mass of this analysis was calculated to be 0.0051, and together with the clearly observable superimposition of the fits and data presented in Fig. 2B, this indicates a superior fit to the model. Similar data were obtained over a protein concentration range of 0.25–2.0 mg/ml, demonstrating that the effect is not concentration-dependent. Assuming a prolate ellipsoidal shape calculated using the method (29), the axial ratio is estimated to be 5.4, indicating that in aqueous solution, Tom70\(^{51–617}\) is 5.4 times longer than it is wide. Sedimentation equilibrium experiments were also performed on Tom70\(^{51–617}\) under the same solution conditions as employed for the sedimentation velocity analyses (Fig. 3). From Equation 1, the equivalent molar mass of Tom70\(^{51–617}\) at an initial concentration of 1.5 mg/ml was calculated to be 56.9 kDa (data not shown). This is in good agreement with the monomeric mass of the protein (65.9 kDa) and is consistent with the sedimentation velocity results (Fig. 2C).

Comparison of Tertiary Structure and Stability by Spectroscopy—To further characterize the structure of Tom70\(^{51–617}\), equilibrium unfolding data derived from intrinsic tryptophan fluorescence were combined with data on global unfolding of the secondary structure, as provided by far-UV CD analysis. The fluorescence emission spectra of Tom70\(^{51–617}\) (Fig. 4A) shows an emission maximum at 333 nm, which is typical of a buried tryptophan residue. Upon the addition of 6 M urea, the emission maximum red-shifted to 353 nm as well as displaying a concomitant decrease in emission intensity. To excite both tyrosine and tryptophan residues, the excitation wavelength was set at 275 nm. There was a complete absence of tyrosine emission at 303 nm in the spectra, although Tom70\(^{51–617}\) has 21 tyrosine residues (Fig. 4B). When Tom70\(^{51–617}\) was unfolded in the presence of 6 M urea, tyrosine fluorescence becomes visible at 303 nm. This is a clear indication of high resonance energy transfer from tyrosine to tryptophan in natively folded Tom70\(^{51–617}\).

The urea-induced unfolding profiles for Tom70\(^{51–617}\), as monitored by changes in fluorescence emission intensity at 333 nm, are shown in Fig. 5. The profiles were identical over a 10 ns protein concentration range (30–300 µg/ml) and were fully reversible. There was a single transition from fully folded to unfolded protein with Tom70\(^{51–617}\) centered around 2.8 M urea (Table I). The protein was fully unfolded when urea concentration exceeded 4 M. The lack of a concentration-dependent step in the unfolding pathway further suggests that the native state of Tom70\(^{51–617}\) is monomeric.

The second transition resulted in a complete loss of secondary structure when urea concentration exceeded 4 M (Fig. 6, inset). The formation of this intermediate was shown to be highly reproducible over a 5× protein concentration range (50–250 µg/ml). As the midpoints for the structural transitions observed via CD (1 and 3.4 M) were not the same as for the transition monitored by fluorescence (2.7 M), it is clear that at least two intermediate species are formed during the unfolding of Tom70\(^{51–617}\). Therefore, the minimal pathway is N ↔ I\(_1\) ↔ I\(_2\) ↔ U. In both CD and fluorescence-monitoring unfolding experiments, Tom70\(^{51–617}\) unfolding was shown to be fully reversible. The data from both experiments were therefore combined and analyzed using either a two- or a three-state model, respectively (Table I).

The thermal stability of Tom70\(^{51–617}\) was also determined by monitoring the change in the secondary structure by circular dichroism. As this denaturation is irreversible, it is unable to be fitted to a thermodynamic model. However, the raw data curve shows that Tom70 unfolds via two transitions (Fig. 7) with midpoints (T\(_m\)) of 41 ± 1 and 52 ± 1 °C. The presence of two transitions indicates the formation of an intermediate species, in good agreement with the urea titration data obtained by CD analysis. This profile was highly reproducible and independent of the heating rate or protein concentration.

| Parameters for the two- and three-state fits to the denaturation transition curves | Two state analysis | Three state analysis |
|---|---|---|
| ΔG\(_F\) → U | m\(_F\) → U | D\(_{50\%}\) F → U | ΔG\(_F\) → I | m\(_F\) → I | D\(_{50\%}\) F → I | ΔG\(_I\) → U | m\(_I\) → U | D\(_{50\%}\) I → U |
| Fluorescence intensity | kcal/mol | kcal/mol | m | kcal/mol | kcal/mol | m | kcal/mol | kcal/mol | m |
| 0.03 mg/ml | 3.17 ± 0.02 | 1.15 ± 0.08 | 2.7 | 2.04 ± 0.1 | 1.94 ± 0.1 | 1.05 | 2.3 ± 0.1 | 2.2 | 1.04 |
| 0.3 mg/ml | 3.50 ± 0.06 | 1.2 ± 0.10 | 2.9 | 3.50 ± 0.1 | 2.1 | 1.62 ± 0.1 | 3.3 |
| Far-UV CD | | | | | | | | | |
| 0.05 mg/ml | | | | | | | | | |
| 0.25 mg/ml | | | | | | | | | |

DISCUSSION

Much of the recent study of helical repeat proteins has focused on the design of “idealized” motifs: artificial proteins in which large scale sequence alignment techniques have been employed to determine the most common residue at each position of an otherwise highly degenerate consensus sequence. Such studies have been performed on ankyrin repeats (35); leucine-rich repeats (36); and TPR repeats (37) and have given insight into the global characteristics of each motif. However, very little work has been done on the biophysical properties of naturally occurring TPR arrays. In this study, we present a focused biophysical and spectroscopic analysis of the TPR arrays of Tom70. Our study is also the first such analysis of a component of the mitochondrial protein import machinery.

The underlying appeal of designing idealized helical repeat arrays is their propensity to exhibit high thermal stability. However, ironically, to date, similar studies on naturally occurring helical repeat arrays have often displayed thermal instability. For example, studies involving an idealized ankyrin helical repeat (an α-helix/β-hairpin/α-helix repeat) showed that the stability of the protein tended to increase as more and more repeats were added, so much so that the T\(_m\) of an artificial 4× ankyrin repeat was 81 °C (35). However, biophysical studies of naturally occurring 4× ankyrin repeat proteins, p16\(_{INK4d}\) and myotrophin, yielded T\(_m\)S of 44 and 53 °C, respectively (38–40). More strikingly, Notch, a receptor with six functional ankyrin repeats, exhibits a very high T\(_m\) of 95 °C.

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TABLE I

![Table I](https://www.jbc.org/content/106/1/46452.full)
repeats, has a $T_m$ of just 45 °C (41). So far, this disparity between idealized and natural stabilities is yet to be explained.

The thermal unfolding data presented in this study show that this inconsistency also applies between idealized and naturally occurring TPRs, with the observation that the thermal unfolding midpoint of Tom7051–617 occurs at 41 °C, as compared with 83 °C as seen in an idealized 3×TPR repeat (37). This instability might be vital for the function of the receptor, with Tom70 presumably needing to display some plasticity in order for it to recognize the wide catalogue of sequences found within the mitochondrial protein ligands.

The unfolding pathway of Tom7051–617 also contrasts that observed in idealized helical arrays in that it unfolds via at least one intermediate. The N $\leftrightarrow$ I$_1$($\leftrightarrow$I$_2$) $\leftrightarrow$ U unfolding pathway of Tom70 was evident in both urea and thermally induced denaturation assays, as observed via CD, with I$_1$ visible at 1 M urea. All intermediates observed were found to be reproducible over a 10-fold concentration range, with urea-induced denaturation also being fully reversible. The transition I$_1$ $\leftrightarrow$ I$_2$ was observed by monitoring tryptophan fluorescence as Tom7051–617 was denatured via urea, with I$_2$ apparent at 2.8 M. The single tryptophan residue of Tom70 resides in TPR1 (residue 124), and during unfolding, has an increase in solvent exposure. As there is no significant loss of secondary structure associated with this transition, its presence could be a result of localized unfolding around TPR1 or of Tom7051–617 moving from a semiglobule to a more extended shape.

The presence of an intermediate species during thermal un-
folding is intriguing and suggests that the protein consists of independent domains with significantly different stabilities. This is supported by previous work which showed that idealized TPR motifs consisting of one, two, or three TPR motifs had midpoints of thermal denaturation of 49, 74, and 83 °C, respectively, which suggests that there is a correlation between the number of repeats and stability (37). As Tom7051–617 has two sets of repeats, one with two TPR motifs and the second with five motifs present (16), it is possible that the presence of an intermediate species reflects the different stabilities of these structures. Given the “nonglobular” asymmetry of Tom70 and the low amount of coiling in the terminal regions of Tom70, this hypothesis seems more plausible than conventional unfolding of a hydrophobic core domain following the unfolding of terminal random or near random coil regions. In keeping with the hypothesis, the presence of intermediate species. The first transition (N → I1) resulted in a loss of secondary structure, whereas the transition from I1 to I2 was only observed by fluorescence spectroscopy.

It is thought that the apparent stability of idealized repeat arrays stems from the cooperative nature of array folding and unfolding, thus explaining why unfolding profiles of idealized TPR motifs display a simple N ↔ U pattern with a high midpoint (37). We hypothesize that the presence of an intermediate in the unfolding pathway of Tom7051–617 indicates that it has two separate TPR arrays, capable of folding and unfolding independently, with presumably separate functions. This correlates well with the primary structure of Tom70, with TPRs 1 and 2 separated from TPRs 3–7 via a 233-residue linker region. TPRs 1 and 2 are required for binding cytoplasmic Hsp70 (9), and 2 separated from TPRs 3–7 via a 233-residue linker region. Moreover, it is generally accepted that a minimum of three stability (37). As Tom7051–617 has two number of repeats and stability (37). As Tom7051–617 has two

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