Tracking the Unfolding Pathway of a Multirepeat Protein via Tryptophan Scanning

EVIDENCE OF LOCALIZED INSTABILITY IN THE MITOCHONDRIAL IMPORT RECEPTOR Tom70®

Simon R. Bushell1, Stephen P. Bottomley2, Jamie Rossjohn3, and Travis Beddoe1,4

From the 1Protein Crystallography Unit, Australian Research Council Centre of Excellence in Structural and Functional Microbial Genomics and the 2Department of Biochemistry and Molecular Biology, School of Biomedical Sciences, Monash University, Clayton, Victoria 3800, Australia

The tetratricopeptide repeat (TPR) is a degenerate 34-amino acid repeating motif that forms a repeating helix-turn-helix structure and is a well characterized mediator of protein-protein interactions. Recently, a biophysical investigation on one naturally occurring TPR protein, Tom70, found that the mitochondrial receptor displayed an unusual three-state unfolding pathway, distinct from the two-state model usually displayed by TPR proteins. To investigate this unusual behavior, we undertook a tryptophan-scanning analysis of Tom70, where both native and engineered tryptophan residues are used as fluorescent reporters to monitor the range of local and global unfolding events that comprise the unfolding pathway of Tom70. Specifically, seven Tom70 variants were constructed, each with a single tryptophan residue in each of the seven TPR repeats of Tom70. By combining equilibrium and kinetic fluorescent unfolding assays, with circular dichroism experiments, our study reveals that the unusual folding pathway of Tom70 is a consequence of the unfolding of two separate, autonomous TPR arrays, with the less stable region appearing to account for the local structural stability of Tom70.

Since it was first identified in 1990 (1), tetratricopeptide repeat (TPR)® motifs have been found in over 300 different proteins (2, 3). Approximately 20 structures of proteins containing TPR motifs have been solved, revealing the motif to consist of a 34-amino acid-long pair of antiparallel α-helices, linked by a short loop region (4). TPRs are often further arranged into large superhelical arrays of between 3 and 20 repeats that provide a contiguous surface by which the repeats can mediate protein-protein interactions.

Investigations into the biophysical characteristics of TPR motifs have focused on the properties of artificial motifs constructed from alignment-derived consensus sequences (5). Such studies have also been performed on other helical repeating motifs, such as ankyrin (6) and leucine-rich repeats (7). These studies have revealed that each separate TPR is intrinsically stable as a result of favorable hydrogen bonding between conserved residues, with stability increasing as more repeats are added to an array. Thermal midpoints of unfolding have been reported as high as 83 °C for a three times artificial TPR repeat (8).

This comparative stability and the apparent ease with which these repeating sequences evolve (9) have meant that TPRs modulate the function of a diverse array of proteins. One such protein, Tom70, is a receptor of the TOM complex, an oligomeric translocase located on the outer membrane of mitochondria. The function of Tom70 is modulated by its complement of at least seven TPR motifs, through which it recognizes Hsp70-bound import-competent mitochondrial proteins with internal noncanonical targeting sequences. Upon its recognition of internal targeting sequences, Tom70 helps facilitate the import of mitochondrial protein through the general import pore of the TOM complex (10). The Tom70 N-terminal TPR motifs (TPRs 1 and 2) are thought to bear a structural and mechanical similarity to that of Hop, a co-chaperone that uses its own TPR motifs to coordinate with the conserved C-terminal aspartate of Hsp70 (11, 12). With this region acting as a clamp, Tom70 recognizes the mitochondrial precursor payload of Hsp70 via its C-terminal TPR repeats (residues 397–617), (13, 14). The roles of any putative TPRs in the linker region between these two arrays remain unknown, but it is possible that they contain additional binding sites for both mitochondrial precursors and other subunits of the TOM complex.

Whereas previous biophysical investigations of TPR repeats have focused on idealized artificially created TPR arrays (5), Tom70 represents an ideal naturally occurring model from which physiological data can be derived. As a member of the TPR superfamily, Tom70 displays several unusual characteristics that warrant further investigation. Bioinformatic analysis of the primary structure of Tom70 yields suggests that its TPR repeats are organized in a 2:5 arrangement (15), an unusual composition, considering most other TPR-containing proteins have just a single array.
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Furthermore, TPR arrays generally utilize a two-state model of folding and unfolding (8); however, it was recently demonstrated that Tom70 unfolds via a three-state pathway (N → I → U) (16). The presence of an unfolding intermediate suggested that each of the two known TPR arrays of Tom70 undergo a separate unfolding event as the protein is denatured. The same study showed that Tom70 does not display the same stability that was seen in their engineered counterparts, with thermal unfolding midpoints of 41 and 53 °C.

In order to explain some of these unusual biophysical properties of Tom70 as well as to further characterize the receptor as a naturally occurring TPR protein, we employed a tryptophan scanning approach to identify what parts of the protein were unfolding. In this study, single tryptophan residues were engineered into a variant of Tom70 lacking its natural tryptophan (Trp124). We exploited the fact that tryptophan emissions in wavelength in proportion to their solvent exposure. Therefore, by monitoring the emission spectra of these engineered Trp-substituted proteins, we were able to derive site-specific data on the conformational changes involved in Tom70 unfolding (17–20). This approach represents an ideal spatial and temporal tool for defining the range of localized unfolding events that comprise the Tom70 unfolding pathway.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—All Tom70 proteins were recombinantly expressed with a C-terminal His tag and purified via Ni2+-nitrilotriacetic acid affinity chromatography. The eluent was concentrated via anion exchange chromatography using a Q-Sepharose column. The protein underwent final purification and buffer exchange using a calibrated Superdex 200 16/60 column (GE Health Sciences). All proteins were stored 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 the absorbance at 280 nm, using an extinction coefficient of 35,250 M–1 cm–1. In each case, 4–6 mg of protein was yielded per liter of media.

Site-directed Mutagenesis—All site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene) as per the manufacturer’s instructions. Potential variants were transformed into XL10 Escherichia coli cloning strain and sequenced at the Monash Micromon DNA sequencing facility. Plasmids from successful variants were transformed into BL21(DE3) E. coli expression strain and purified as described above.

Circular Dichroism—Circular dichroic 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-s/poin signal averaging. θ 222 measurements were made with the signal averaged over 15 s. The concentration of the protein was 0.15 mg/ml (2 μM) with a 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 (21). All unfolding data were fitted to either a two- or three-state unfolding model as described previously (22, 23).

Spectrofluorometry 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, and a scan speed of 10 nm/min was used. Unless otherwise stated, an excitation wavelength of 295 nm was used for all experiments.

RESULTS

Generation and Biophysical Analysis of Tom70W124F—Wild-type Tom70 contains a single tryptophan residue at residue 124. This would place the residue at position 26 in TPR 1, as determined by Pfam. Fig. 1 shows an alignment of the seven known TPR motifs of Tom70 as well as a previously derived consensus sequence (2). The alignment shows that the tryptophan of the wild type is at a position usually occupied by leucine, lysine, or glutamate. Using site-directed mutagenesis, Trp124 was conservatively mutated to a phenylalanine, ensuring that any emission signal detected when the protein is excited at 295 nm will arise from a single tryptophan introduced into each TPR motif.

To ensure that the folding of Tom70W124F was not adversely affected by the mutation, a series of spectroscopic and spectrofluorometric experiments were performed to ensure that the protein had similar folding properties and stability to the wild type. Far-UV CD spectra showed that Tom70W124F had a double minimum at 222 and 208 nm, indicating that the protein’s highly helical secondary structure had not been disrupted. Thermal denaturation experiments showed that the protein had thermal stability identical to that of wild type, with midpoints of 41 and 53 °C. Chemical denaturation was also monitored spectrofluorometrically by measuring the shift in emission wavelength when the tyrosine residues of Tom70 were excited at 280 nm. The midpoint of unfolding was 1.1 M urea, identical to that seen in the wild type (16), suggesting that the W124F substitution had not noticeably altered the folding or stability of Tom70.

Generation of Trp-substituted Tom70 Variants—We took a consensus-based approach in determining where best to introduce tryptophan residues in each TPR motif. According to a published consensus sequence, shown in Fig. 1B, position 4 of a TPR motif is usually a tryptophan, a tyrosine, or a leucine (2). The alignment of the Tom70 TPR motifs, in the same figure, shows that this consensus holds true in this case. With a view to maintaining the structural integrity of the protein, we reasoned that a conservative substitution for tryptophan in position 4 of each TPR motif would provide consistency in our results as well as conserve the properties of the TPR motif, making it less likely that the folding and structure of the motif would be perturbed.

With Tom70W124F as a template, site-directed mutagenesis was employed to mutate position 4 of each TPR motif (with the
exception of TPRs 2 and 5) to a tryptophan. In the case of TPR 2, a Y135W mutation would have placed a tryptophan in close proximity to the former position of the wild-type tryptophan. To ensure a separate reading for TPR 2, the tryptophan was introduced at a position similar to that of the wild-type tryptophan in TPR 1. In the case of TPR 5, the presence of two proline residues in the first four residues would suggest that this TPR motif is highly atypical, so a nearby phenylalanine residue (Phe470; position 6 of the putative motif) was changed. The final series of tryptophan-substituted variants generated from the Tom70W124F template is as follows: TPR 2, S155W; TPR 3, Y400W; TPR 4, Y434W; TPR 5, F470W; TPR 6, L508W; TPR 7, K545W. In addition, we used wild-type Tom70 to analyze the movements in the region around TPR 1.

**Trp-substituted Tom70 Variants Display the Same Folding Properties as Wild Type**—Tom70 Trp-containing variants were all purified to homogeneity, with protein yield in all cases being 3–6 mg/liter of media (comparable with wild type). All proteins demonstrated the same elution profile when purified with a Superdex 200 size exclusion column ($V_e/H = 72$ ml). This would initially indicate that each variant is folded similarly to that of the wild type with no change to the protein’s hydrodynamic radius. It has been previously demonstrated that the comparatively late elution of Tom70 from the Superdex 200, for a 70-kDa protein, is due to its high asymmetry and not homodimerization (16).

To further confirm the correct folding of each variant, the far-UV CD spectra were determined for each protein. All spectra were identical to the wild type spectra, with double minima at 222 and 208 nm with similar mean residue ellipticity, indicating that the tryptophan substitutions do not alter the secondary structure. Thermal melts of each protein, monitored at 222 nm via far-UV CD, demonstrated that there is no appreciable change in each vari-
ant’s stability, with variants showing midpoints of unfolding at \( \sim 41 \) and 53 °C, as was also observed with the wild-type protein (data not shown).

When Tom70 was denatured with urea, the resultant unfolding curve (as monitored at 222 nm by CD) displayed a three-state unfolding pathway (16). To confirm that the presence of the engineered tryptophan residues does not perturb this behavior, each variant was subjected to similar unfolding assays. The overlaid unfolding curves can be seen in Fig. 2. The curves were fitted to a three-state unfolding model with the curve parameters comprising Table 1. All of the proteins displayed unfolding midpoints at \( \sim 1 \) and 3.3 M urea. The concordance of all of the equilibrium CD data indicates that all of the variants unfold via a similar pathway and that the tryptophan substitutions did not significantly affect the protein’s native fold or the stability of either the native or intermediate conformations. Accordingly, conclusions drawn from the folding of wild-type Tom70 will also hold for the Trp-substituted variants.

**Identification of a Separately Folded Region**—When excited at 295 nm, tryptophan emits fluorescent radiation at a wavelength between 300 and 400 nm. The wavelength of maximum emission is proportional to the solvent exposure of the particular residue, with wavelength maxima \( \sim 350 \) nm, indicating that the residue is fully exposed. Thus, tryptophan represents an ideal marker in resolving which parts of a protein are unfolding (17–20).

When Tom70 unfolding is monitored by measuring the shift in emission wavelength, an unfolding midpoint of 2.8 M urea is observed (16). This midpoint does not correspond to the unfolding events observed when unfolding is monitored by

**TABLE 1**

| TPR Mutation | \( \Delta G_{F \rightarrow I} \) | \( D_{F \rightarrow I}^{50\%} \) | \( \Delta G_{I \rightarrow U} \) | \( D_{I \rightarrow U}^{50\%} \) |
|--------------|----------------|----------------|----------------|----------------|
| 1 Wild type  | 2.05 ± 0.07 | 1.10 | 5.50 ± 0.06 | 3.16 |
| 2 S155W      | 2.19 ± 0.08 | 1.17 | 5.62 ± 0.08 | 3.23 |
| 3 Y400W      | 2.33 ± 0.06 | 1.25 | 5.34 ± 0.07 | 3.07 |
| 4 F434W      | 2.51 ± 0.08 | 1.35 | 5.39 ± 0.08 | 3.10 |
| 5 F470W      | 2.17 ± 0.03 | 1.16 | 5.31 ± 0.03 | 3.05 |
| 6 L508W      | 2.16 ± 0.14 | 1.16 | 5.57 ± 0.14 | 3.20 |
| 7 K545W      | 2.19 ± 0.03 | 1.18 | 5.51 ± 0.03 | 3.17 |

**FIGURE 3.** Fluorescence analysis of the urea induced unfolding of Tom70 and its single tryptophan variants. The equilibrium unfolding curves for each protein were determined by measuring the changes in tryptophan emission intensity when excited at 295 nm as a function of urea concentration. Data points in each graph are represented by hollow squares, with the fitted curve represented by a dashed line. All curves were fitted to a two-state unfolding model, as described under “Experimental Procedures” with unfolding parameters tabulated in Table 2.
far-UV CD, with both the N → I and I → U transitions having midpoints of 1.05 and 3.2 M urea, respectively. It was therefore apparent that structural changes associated with these transitions were occurring elsewhere within the protein.

The local changes in tertiary structure at each TPR motif were monitored by measuring the decay of emission signal at the \( \lambda_{max} \) of each variant. The resultant unfolding curves of each protein are shown in Fig. 3, with thermodynamic parameters detailed in Table 2. Unlike the two transitions observed with far-UV CD, the unfolding of each single tryptophan protein occurs in a single cooperative transition, indicative of a two-state unfolding event. The curves show two distinct midpoint groupings. The less stable group comprises the variants with mutated tryptophans in TPRs 4–6, with unfolding midpoints centered around 1.7, 1.6, and 1.1 M urea, respectively. The remaining variants have midpoints that cluster around 3.0 M urea.

**Kinetic Unfolding Experiments Confirm Metastable Region**—In addition to the equilibrium unfolding experiments performed above, the unfolding kinetics of each protein was also determined using stopped flow. In these experiments, folded Tom70 was mixed with 8 M urea, and the change in fluorescence intensity was observed. All variants displayed rapid unfolding with unfolding complete within 1 s for all of the proteins. Fig. 4 shows the stopped-flow-determined unfolding curve for wild-type Tom70 as well as the curves for the variants representing TPR 2 and TPR 4, the slowest and quickest unfolding variants, respectively. All of the curves could be adequately described by a single exponential function; the rate constants for unfolding, \( k_{FU} \), for all proteins are shown in Table 2. The unfolding of the TPR 4 variant was extremely rapid, with a rate of 253 s\(^{-1}\), followed closely by TPR 5, with a rate of 55 s\(^{-1}\). The slowest rate was observed for TPR 2, with a rate of 2.1 s\(^{-1}\), correlating well with its position as the most stable variant as determined in the fluorescent studies.

The clustering of the unfolding rate constants and the equilibrium unfolding midpoints into two groups correlates with our hypothesis that the region encompassing TPRs 4–6 is a distinctly less stable region than the rest of the protein. In both cases, the region around TPR 2 was found to be among the most stable.

**DISCUSSION**

It has previously been hypothesized that the Tom70 complement of TPR repeats are arranged in autonomously folding arrays (16). The far-UV CD spectrum of Tom70 indicates that it is highly \( \alpha \)-helical, which would suggest that the bulk of its structure lies within these TPR motifs. When urea-induced changes in secondary structure are monitored via CD, Tom70 demonstrates a three state unfolding curve (N → I → U). Given that it has previously been shown that TPR motifs usually unfold via a two-state pathway, it suggests that this intermediate represented a separately folded array that displayed less stability than other such arrays within the protein. By employing tryptophan-scanning mutagenesis, we have been able to show that the unfolding midpoints of each of the Tom70 TPR motifs cluster into two distinct groups, reflecting the differential unfolding of distinct parts of the protein.

This is surprising, since most TPR proteins only contain their motifs in a single array. A notable exception to this trend is the peroxisomal receptor PEX5, whose TPR arrays appear to be arranged in a clamp type arrangement (24). Whereas this protein has a solved crystal structure, there is no biophysical study to indicate whether it unfolds through a three-state pathway. Under the current model of the mode of action of Tom70, the receptor clamps the chaperone component of the Hsp70-precur- sor complex via TPR 1 and TPR 2, with recognition of the precursor’s targeting sequences facilitated by the C-terminal TPRs (11). It has been shown that a 25-kDa proteolytically stable fragment encompassing residues 247–460 (the area preceding and including TPR 4) is able to bind targeting sequences at a similar capacity to that of the full-length protein (25), indicating that this region might represent the receptor’s major binding site. Given the highly degenerate nature of internal mito-

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**TABLE 2**

Results from Figs. 3 and 4 were fitted to a two-state unfolding analysis as described previously.

| TPR Mutation | \( \lambda_{max} \) (nm) | \( \Delta G_{F \rightarrow U} \) (kcal/mol) | \( m_{F \rightarrow U} \) (M) | \( D_{FU} \) (s) | \( k_{FU} \) (s\(^{-1}\)) |
|--------------|--------------------------|-------------------------------|----------------|----------------|----------------|
| 1 Wild type  | 333 | 3.68 ± 0.3 | 1.31 ± 0.07 | 2.8 | 33.64 ± 2.0 |
| 2 S153W     | 333 | 3.05 ± 0.2 | 1.02 ± 0.16 | 3.0 | 2.097 ± 0.22 |
| 3 Y400W     | 342 | 5.22 ± 0.2 | 1.66 ± 0.09 | 3.1 | 10.87 ± 0.64 |
| 4 F4a34W    | 338 | 1.83 ± 0.1 | 1.14 ± 0.15 | 1.6 | 252.9 ± 44.24 |
| 5 F470W     | 330 | 1.27 ± 0.2 | 0.85 ± 0.15 | 1.5 | 54.59 ± 2.74 |
| 6 L508W     | 340 | 1.47 ± 0.2 | 1.50 ± 0.2 | 1.0 | 6.26 ± 0.82 |
| 7 K545W     | 330 | 2.10 ± 0.2 | 0.81 ± 0.1 | 2.6 | 5.27 ± 0.90 |

**FIGURE 4. Unfolding kinetics of Tom70 and its variants.** The rates of unfolding for all variants were measured in a stopped-flow apparatus as described under “Experimental Procedures.” The curve for wild-type Tom70 (representing TPR 1) is shown here with the curves for variants representing TPRs 2 and 4, which constituted the most stable and unstable repeats, respectively. Values for \( k_{FU} \) were calculated by fitting the data to a one-phase exponential decay function. \( k_{FU} \) values for all variants are included in Table 2.
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...targeting sequences, one might expect this region to display a high degree of plasticity in order to facilitate such binding. The similarly low stability of TPRs 5 and 6 might represent a mechanical means of improving specificity or a means of facilitating precursor transfer to downstream components of the TOM complex.

Conversely, the region around TPR 2 appears to be the most stable, as reflected by the high equilibrium unfolding midpoint (3.0 M) and the low $k_{\text{NU}}$ when unfolding is observed via stopped flow experiments. This region is thought to constitute a binding site for the highly conserved C-terminal region of Hsp70 (11). This stability would therefore be crucial in the specificity of this binding event. The comparative stability of both TPR 3 and TPR 7 could serve to constrain the receptor's instability to just TPR 4–6. Such a mechanism is not without precedent; stabilizing helices have been observed and employed in a number of helical repeat systems (8).

The unfolding kinetics show general agreement with the clustering of midpoints seen in fluorometric experiments and correlates well with the observed relative order of TPR stability. The values for $k_{\text{NU}}$ in the variants representing TPRs 4–6 were generally lower than that observed in other repeats. The reason for the variance in the order of stability is probably due to the slight effects a mutation might have on the TPR stability being amplified by the high speeds at which Tom70 unfolds in such an experiment.

The variance in stabilities seen in even adjacent TPR motifs seems to indicate that their folding in Tom70 is noncooperative. Furthermore, this variance would suggest that it is not always stability that is the chief advantage that the motif confers upon the protein. In the case of Tom70, the stability varies in accordance with the role the motif plays in the protein's function. The Tom70 TPR motifs display both stability in the recognition of Hsp70 and flexibility in the binding to mitochondrial targeting sequences. It would therefore appear that adaptability is a key asset of the TPR in its role in mediating protein-protein interactions. In this regard, it is of interest to examine how TPRs evolved in systems that involve cross-talk between two different organisms. For example, it has recently been shown that proteins with primitive TPR motifs are secreted by the pathogen Legionella pneumophila to interfere with the host macrophage cell's stress response effectors (26). For such a system to be effective, the adaptors would presumably need to display a balance of stability and flexibility to carry out its role. Given the bacterial origins of the mitochondrion, the evolution of a complex system for maintaining the integrity of the organelle in the face of the transfer of its genes to the nuclear genome could be achieved comparatively quickly (through gene duplication) by using a TPR system. As such, the mitochondrial import system contains two major receptors that utilize TPRs in performing their respective roles (27, 28).

In conclusion, we have demonstrated the effectiveness of using mutated tryptophan residues in observing the myriad of localized unfolding events that comprise a protein's folding pathway. Since the bonds that stabilize TPR-proteins, such as Tom70, are extremely localized when compared with globular proteins, the tryptophan-scanning technique is particularly effective when discerning the pathways of repeat proteins. It has allowed us to identify two regions of Tom70 that fold autonomously (a stable Hsp70-binding region and a less stable region that recognizes mitochondrial targeting sequences) and form a model that explains how their respective stabilities are vital to function.

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