Structural basis for mutation-induced destabilization of profilin 1 in ALS

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Et al.
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Mutations in profilin 1 (PFN1) are associated with amyotrophic lateral sclerosis (ALS); however, the pathological mechanism of PFN1 in this fatal disease is unknown. We demonstrate that ALS-linked mutations severely destabilize the native conformation of PFN1 in vitro and cause accelerated turnover of the PFN1 protein in cells. This mutation-induced destabilization can account for the high propensity of ALS-linked variants to aggregate and also provides rationale for their reported loss-of-function phenotypes in cell-based assays. The source of this destabilization is illuminated by the X-ray crystal structures of several PFN1 proteins, revealing an expanded cavity near the protein core of the destabilized M114T variant. In contrast, the E117G mutation only modestly perturbs the structure and stability of PFN1, an observation that reconciles the occurrence of this mutation in the control population. These findings suggest that a destabilized form of PFN1 underlies PFN1-mediated ALS pathogenesis.

Significance

Mutations in profilin 1 (PFN1) were recently shown to cause amyotrophic lateral sclerosis (ALS); however, little is known about the pathological mechanism of PFN1 in disease. We demonstrate that ALS-linked mutations cause PFN1 to become destabilized in vitro and in cells, likely through a mechanism that involves mutation-induced cavities within the protein core. Changes in protein stability due to disease-causing mutations can play a pivotal role across different disease mechanisms. The destabilized mutant-PFN1 species identified here can serve as an upstream trigger for either loss-of-function or gain-of-toxic-function mechanisms and thus emerges from these studies as a pertinent therapeutic target for the incurable disease ALS.


The authors declare no conflict of interest.

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Data deposition: The crystallography, atomic coordinates, and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 4X1L, 4X1M, and 4X2S).

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here and applied to all PFN1 variants (Materials and Methods). PFN1 C71G was found to be highly prone to aggregation in E. coli, consistent with observations that this variant exhibited particularly low solubility in mammalian cells (2), and therefore was isolated from inclusion bodies (Materials and Methods). The biochemical properties of PFN1 C71G purified from inclusion bodies are indistinguishable from PFN1 C71G purified from the soluble lysate of E. coli as determined by several assays (Fig. S1), providing confidence that PFN1 proteins purified by these two methods can be directly compared.

To examine the stability of PFN1 proteins, fluorescence from trytophans (W4 and W32) in PFN1 WT and ALS-linked variants was measured as a function of increasing urea concentration (Fig. L4). To ensure reversibility, the reciprocal analysis was also performed, where denatured PFN1 proteins in urea were refolded upon dilution with buffer (Fig. S2 A–E). Only one transition was observed between the folded or native (N) and unfolded (U) states for all PFN1 proteins, indicative of a two-state (N ↔ U) unfolding mechanism. This two-state unfolding model was further substantiated with an unfolding study of two PFN1 proteins (WT and M114T) using CD spectroscopy (Fig. S2F). The following thermodynamic parameters were determined by fitting the fluorescence data to a two-state folding model: apparent ΔG°, the free energy of folding; m, the denaturant dependence of ΔG°; and Cm, the midpoint of the unfolding transition (Table 1). Both ΔG° and Cm were reduced for ALS-linked variants relative to PFN1 WT, particularly for the PFN1 variants C71G, M114T, and G118V, indicating these variants are severely destabilized compared with PFN1 WT (Fig. L4 and Table 1). Differential scanning fluorimetry (DSF) with SYPRO Orange, a fluorescent indicator of hydrophobic regions exposed upon protein unfolding, was used next to determine the apparent melting temperature, Tm, for all PFN1 proteins used in this study (15). Consistent with the chemical denaturation results, all ALS-linked variants except E117G exhibited a Tm that was at least 10 °C lower than WT (Fig. L5 and Table 1). Based on the denaturation studies, C71G emerges as the most destabilizing mutation in the context of PFN1, whereas the E117G mutation has a relatively modest impact on PFN1 stability.

**Table 1. Summary of experimental stability and binding measurements for PFN1 variants**

<table>
<thead>
<tr>
<th>Variant</th>
<th>ΔG°, kcal·mol⁻¹</th>
<th>m, kcal·mol⁻¹·M⁻¹</th>
<th>Cm, M</th>
<th>Protein alone + 4 mM proline</th>
<th>Tm, °C</th>
<th>Binding to poly-L-proline¹·²</th>
<th>Kₚ, µM</th>
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<tr>
<td>WT</td>
<td>7.04 ± 0.49</td>
<td>2.25 ± 0.16</td>
<td>3.13 ± 0.31</td>
<td>54.68 ± 0.04</td>
<td>57.25 ± 0.03</td>
<td>463 ± 26</td>
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<tr>
<td>C71G</td>
<td>1.89 ± 0.70</td>
<td>1.95 ± 0.40</td>
<td>0.97 ± 0.41</td>
<td>34.60 ± 0.03</td>
<td>39.96 ± 0.03</td>
<td>687 ± 77</td>
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<tr>
<td>M114T</td>
<td>3.51 ± 0.40</td>
<td>2.51 ± 0.24</td>
<td>1.40 ± 0.21</td>
<td>42.62 ± 0.03</td>
<td>46.52 ± 0.02</td>
<td>572 ± 23</td>
<td></td>
</tr>
<tr>
<td>E117G</td>
<td>6.90 ± 0.74</td>
<td>2.49 ± 0.26</td>
<td>2.77 ± 0.42</td>
<td>51.05 ± 0.04</td>
<td>53.78 ± 0.03</td>
<td>407 ± 27</td>
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<tr>
<td>G118V</td>
<td>3.70 ± 0.44</td>
<td>2.20 ± 0.23</td>
<td>1.68 ± 0.26</td>
<td>42.84 ± 0.04</td>
<td>46.92 ± 0.04</td>
<td>397 ± 40</td>
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</table>

*Errors are shown as SD.
†Errors are shown as SE.
‡Kₚ values are reported in terms of proline residues.

Fig. 1. ALS-linked mutations destabilize PFN1. Chemical and thermal denaturation studies reveal that ALS-linked variants C71G, M114T, and G118V, but not E117G, are severely destabilized relative to PFN1 WT. (A) Equilibrium unfolding curves for PFN1 WT and ALS-linked variants generated by measuring the intrinsic tryptophan fluorescence of the indicated protein equilibrated in increasing concentrations of urea. Data were processed to obtain the center of mass (COM) of the emission spectrum and then fit to a two-state model for protein folding. The resulting fits are displayed as solid lines. The corresponding thermodynamic parameters obtained from the fitted data are shown in Table 1. (B) Thermal denaturation profiles of PFN1 proteins measured by SYPRO Orange fluorescence as a function of increasing temperature were used to determine the apparent Tm, which is the temperature corresponding to 0.50 fluorescence signal as denoted by the intersection of the dashed lines for each curve.

**ALS-Linked PFN1 Exhibits Faster Turnover in a Neuronal Cell Line.** The turnover rate for proteins with destabilizing mutations is often faster relative to their WT counterparts, generally because destabilized proteins are misfolded and targeted for degradation by the cellular quality control machinery (16). To determine whether the results of our in vitro denaturation studies extend to a cellular environment, V5-tagged PFN1 variants were transiently transfected into human neuronal SKNAS cells, and PFN1 turnover was assessed by tracking V5-PFN1 protein expression over a 12.5-h time course in the presence of cycloheximide. At the start of the experiment (t = 0) of the cycloheximide time course), all V5-tagged PFN1 variants were expressed at similar levels except that V5-PFN1 C71G, M114T, and G118V partitioned into the insoluble fraction (Fig. 2 A and B) as reported previously (2). The turnover of both PFN1 C71G and M114T occurred significantly faster than that of PFN1 WT. As early as 2.5 h, the majority of PFN1 C71G and M114T within the soluble fraction had already degraded (Fig. 2 A and C). This decrease in soluble PFN1 content was not simply due to further PFN1 aggregation, which could confound our analysis, as evidenced by the concomitant clearance of PFN1 from the insoluble fraction at the early time points of cycloheximide exposure (Fig. 2B). The faster turnover of PFN1 C71G and M114T in cells closely correlates with their reduced stabilities in vitro, confirming the destabilizing effect of the C71G and M114T mutations. We note that the turnover of PFN1 C71G was faster in the soluble fraction compared with the insoluble fraction (Fig. S3), likely because clearance of insoluble cellular aggregates by the quality control machinery is less efficient compared with the turnover of smaller, soluble species (17). Although PFN1 G118V was destabilized to a similar degree as M114T in vitro, the turnover of this variant within the soluble fraction seemed slower in cells (Fig. 2C), which may reflect a stabilizing effect of other proteins and/or factors that interact with PFN1 in the cellular milieu (4), or that this variant is not properly handled by the quality control machinery in the cell. In fact, we detected a low level of insoluble PFN1 G118V that persisted throughout the 12.5-h time course (Fig. 2B and Fig. S3).

**ALS-Linked Mutations Induce a Misfolded Conformation Within PFN1.** We reasoned that ALS-linked variants must undergo some degree of structural or conformational change to account for their destabilization. However, ALS-causing mutations did not perturb...
the secondary structural elements of PFN1 as determined by CD spectroscopy (Fig. S4), and the fact that similar m values were determined for all PFN1 variants by the urea denaturation analysis (Table 1) (18). To probe further for potential structural differences between PFN1 WT and ALS-linked variants, these proteins were subjected to native gel electrophoresis, a biochemical technique capable of detecting conformational differences between misfolded variants and their WT counterparts (19). PFN1 WT and E117G migrated predominantly as single, distinct bands with similar mobility, whereas multiple bands of slower mobility were observed for PFN1 variants C71G, M114T, and G118V (Fig. S5A). The slower mobility bands likely reflect the larger hydrodynamic volume due to partial unfolding of these variants. In addition, PFN1 C71G, M114T, and G118V produced relatively large-molecular-weight species that were retained in the stacking gel and unable to electrophorese through the separating native gel but were resolublized under conditions used for the denaturing gel (Fig. S5A). Analytical size-exclusion chromatography revealed that all PFN1 proteins eluted as expected for soluble, monomeric PFN1 (Fig. S5 B–G). However, despite equal loading of PFN1 proteins onto the analytical size-exclusion column, the peak area corresponding to soluble monomer PFN1 is reduced for ALS-linked variants, particularly for the most aggregation-prone variant, C71G. These data are consistent with a loss of soluble monomer PFN1 in the form of insoluble species that cannot pass through the analytical size-exclusion column filter.

A Source of Mutation-Induced Destabilization Revealed by X-Ray Crystallography of PFN1. Crystal structures of PFN1 proteins were determined to identify regions within mutant PFN1 that are conformationally distinct from PFN1 WT at atomic resolution. PFN1 WT, E117G, and M114T produced crystals that diffracted at relatively high resolution (~2.2 Å; Table S1). The 3D structure of human PFN1 WT agrees well with previously determined structures (20–22). PFN1 WT and E117G crystallized in the same space group, C121, whereas M114T crystallized in the P6 space group, with two molecules (designated as chains A and B) in the asymmetric unit (Table S1).

Fig. 2. ALS-linked PFN1 variants exhibit faster turnover in a neuronal cell line. SKNAS cells transiently transfected with V5-PFN1 constructs were treated with cycloheximide (CHX) for up to 12.5 h, during which time lysates were collected and probed by Western analysis with a V5-specific antibody to assess the rate of PFN1 turnover in cells. (A and B) A representative Western blot analysis of soluble and insoluble fractions from cell lysates demonstrates a decrease in V5-PFN1 protein with time. GAPDH serves a loading control for the soluble fraction. (C) Denaturation analysis of A reveals that the turnover of PFN1 C71G and M114T is significantly faster than that of PFN1 WT. Statistical significance was determined using a two-way ANOVA followed by a Tukey’s post hoc analysis (P < 0.05, ***P < 0.001, *P < 0.0001). Error bars represent SEM. WT and E117G, n = 3; G118V, M114T and C71G, n = 4 independent experiments.

Fig. 3. Superimposition of the crystal structures for PFN1 WT, E117G, and M114T. (A and B) The secondary and tertiary structures for PFN1 WT (green), E117G (mustard), M114T chain A (pink), and B (red) are highly superimposable. For each structure, sticks and spheres denote the side chains and van der Waals radii, respectively, for residues at position 114 and 117. Residue 117 is located within a solvent-exposed flexible loop that has no discernible secondary structure, whereas Met114 is located within a β-sheet toward the interior of the protein. (B) A zoomed cartoon representation showing residues within 4 Å of residue 114. The side chains of these residues are indicated as sticks with nitrogen, oxygen, and sulfur atoms indicated in blue, red, and yellow, respectively. The van der Waals radii of the atoms comprising residue 114 are reduced upon mutation of methionine (green and mustard structures) to threonine (red and pink structures).
ALS-linked PFN1 variants retain the ability to bind poly-proline to the same extent (Fig. 6). Of the four ALS-linked mutants investigated in this study, only the C71G variant that exhibits impaired binding to actin failed to suppress actin polymerization, whereas the H120E variant, which is predicted to form a void in the core of the protein that partially overlaps with the cleft observed in the PFN1 M114T structure (Fig. 7B). Analysis of the binding capacity of our PFN1 proteins for G-actin was measured by comparing their concentration-dependent abilities to suppress spontaneous polymerization of pyrenylidoacetamide-labeled actin monomers (34). This assay is based on the fact that PFN1 binds G-actin and inhibits actin nucleation in the absence of formins (34). This assay is based on the fact that PFN1 binds G-actin and inhibits actin nucleation in the absence of formins (34). We first monitored changes in the intrinsic tryptophan fluorescence of PFN1 as a function of poly-proline peptide concentration (Fig. 5A). Our results revealed that the effect of ALS-linked mutations on the PFN1-poly-proline interaction was modest, because the apparent dissociation constants ($K_d$) were within twofold for all PFN1 proteins in this study (Table 1). In fact, excess concentrations of poly-proline effectively stabilized all PFN1 proteins as determined by DSF, with the largest increase in $T_m$ observed upon poly-proline peptide binding to C71G (Fig. 5B and Table 1). Next, we measured the binding capacity of our PFN1 proteins for G-actin by comparing their concentration-dependent abilities to suppress spontaneous polymerization of pyrenylidoacetamide-labeled actin monomers (34). This assay is based on the fact that PFN1 binds G-actin and inhibits actin nucleation in the absence of formins (34).

As expected, increasing concentrations of recombinant PFN1 WT reduced the rate of actin polymerization, whereas the H120E variant that exhibits impaired binding to actin failed to suppress actin polymerization to the same extent (Fig. 6). Of the four ALS-linked variants, only G118V was defective in suppressing actin polymerization, which was most apparent at the highest concentration of PFN1 used in this assay, although this effect did not reach statistical significance (Fig. 6). These data argue against a general mechanism for PFN1-mediated ALS pathogenesis that involves impaired direct binding between PFN1 and either poly-proline or actin.

Importantly, the X-ray crystal structures reveal a possible mechanism by which ALS-linked mutations destabilize PFN1. Residues Thr90, Met114, and Glu18 contribute to the formation of a surface exposed pocket that was detected using SiteMap (Fig. 7). Mutation of methionine to threonine at position 114 increased the size of this pocket, thereby forming a cleft, because the residues nearby failed to rearrange and compensate for the loss of van der Waals contacts (Fig. 7B). This cleft is expected to exert a destabilizing effect on the native conformation of PFN1 owing to this loss of van der Waals contacts and the reduced hydrophobicity of the threonine side chain relative to that of methionine (11). Moreover, hydrophobic residues that are otherwise buried in the PFN1 WT structure were exposed by the cleft in the PFN1 M114T structure (Fig. 7 and Fig. S9). To investigate the potential impact of the C71G mutation on PFN1 structure, the cysteine side chain of residue 71 was removed to mimic a glycine amino acid in the PFN1 WT structure acid in the PyMOL software (8). Interestingly, this mutation is predicted to form a void in the core of the protein that partially overlaps with the cleft observed in the PFN1 M114T crystal structure (Fig. 7B). Analysis of binding capacity of our PFN1 proteins for G-actin was measured by comparing their concentration-dependent abilities to suppress spontaneous polymerization of pyrenylidoacetamide-labeled actin monomers (34). This assay is based on the fact that PFN1 binds G-actin and inhibits actin nucleation in the absence of formins (34). As expected, increasing concentrations of recombinant PFN1 WT reduced the rate of actin polymerization, whereas the H120E variant that exhibits impaired binding to actin failed to suppress actin polymerization to the same extent (Fig. 6). Of the four ALS-linked variants, only G118V was defective in suppressing actin polymerization, which was most apparent at the highest concentration of PFN1 used in this assay, although this effect did not reach statistical significance (Fig. 6). These data argue against a general mechanism for PFN1-mediated ALS pathogenesis that involves impaired direct binding between PFN1 and either poly-proline or actin.

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PFN1 is located within a solvent-exposed flexible loop, it is difficult to predict whether this mutation propagates structural changes to the same region affected by M114T. We note that the phi and psi angles for Gly118 are in a region of the Ramachandran plot that are generally disallowed for a valine residue, and therefore we speculate that the G118V mutation also induces a conformational change within PFN1 that allows valine to adapt dihedral angles that are energetically more favorable.

Our study also provides insight into the relative pathogenicity of ALS-linked PFN1 variants. The pathogenicity of the E117G variant was called into question after it had been detected in the control population (2, 14, 37, 38). Moreover, this variant exhibited mild phenotypes compared with other ALS-linked PFN1 variants in cell-based functional experiments (2, 7). Here, the E117G mutation had only a modest effect on the stability and structure of PFN1 (Table 1 and Fig. S6), supporting the view that E117G is a risk factor for disease rather than overtly pathogenic (1, 14). Further, the E117G mutation was detected in sporadic ALS and frontotemporal lobar degeneration cases (14, 37–40), consistent with the idea that environmental factors and/or genetic modifiers contribute to PFN1 E117G toxicity. In fact, proteasome inhibition triggered the aggregation of PFN1 E117G (2), suggesting that cellular stress may exacerbate PFN1 misfolding and dysfunction in vivo.

Although the mechanism of PFN1 in ALS has yet to be fully elucidated, the destabilized mutant-PFN1 species identified here can serve as an upstream trigger for either loss-of-function or gain-of-toxic-function mechanisms. Several investigations from cell-based experiments support a loss-of-function mechanism for ALS-linked PFN1 variants with respect to actin binding (2), actin dynamics (2), and stress granule assembly (7). For example, PFN1 variants immunoprecipitated less actin from mammalian cells compared with PFN1 WT (2). Our in vitro results suggest this is unlikely due to a general defect in the inherent ability of mutant PFN1 to directly bind actin (Fig. 6) but may be the consequence of mutant PFN1 being sequestered away from actin and/or engaged in other aberrant interactions within the cell. Moreover, ALS-linked mutations do not simply abrogate the direct-binding interaction between PFN1 and the poly-l-proline motif (Fig. 5A) that is present in many biological PFN1 ligands. These data, however, do not rule out the possibility that mutation-induced misfolding and destabilization culminate in defective actin homeostasis in vivo. PFN1 plays a complex role in actin homeostasis, requiring coordinated interactions between PFN1 and many other cellular factors that ultimately dictate the fate of different actin networks within the cell (41).

The misfolding of PFN1 variants may also induce gain of toxic functions and interactions, the latter via aberrant protein–protein interactions through exposed hydrophobic patches, such as those detected for PFN1 M114T (Fig. S9). Further, the aggregation of PFN1 variants can potentially sequester other vital proteins, including those with poly-l-proline binding motifs (4), culminating in compromised actin and/or cellular homeostasis (6).

Although the downstream effect of ALS-linked PFN1 on actin dynamics and other cellular processes have not been elucidated, our data identify misfolded and destabilized PFN1 as a potential upstream trigger of the adverse events that culminate in ALS, opening new avenues for therapeutic advancement in ALS. One potential direction is the development of pharmacological chaperones (16). For example, small molecules that fill the void formed by the M114T mutation are expected to stabilize the protein (35). Our data with poly-l-proline (Fig. 5B) suggest that small-molecules binding to other regions of PFN1 could also stabilize the protein. We posit that stabilizing mutant PFN1 will restore the normal structure and function of the protein, thereby preventing the pathogenic cascade leading to ALS.

**Fig. 6.** The binding of PFN1 proteins to G-actin. Polymerization of monomeric rabbit muscle actin (3 μM, 5% pyrene-labeled) was monitored in the presence of increasing concentrations of WT or ALS-linked PFN1 variants and used to derive relative rates of polymerization (n = 3). The variant H120E, which is impaired in binding to actin, fails to suppress spontaneous actin polymerization as effectively as WT PFN1. Although G118V is relatively weak in suppressing actin polymerization, the data did not reach statistical significance. Statistical significance was determined using a two-way ANOVA followed by a Tukey’s post hoc analysis. **P ≤ 0.01 for WT vs. H120E at 7 μM concentration. No other significant comparisons with WT were obtained. Other significant comparisons included C71G vs. H120E and E117G vs. H120E (P ≤ 0.05) at 7 μM concentration. Error bars represent SD.**

**Fig. 7.** The M114T mutation causes a surface-exposed pocket to expand into the core of the PFN1 protein. (A) Residues are depicted as described in Fig. 3. The van der Waals radii of residues 90, 114, and 18 are in contact in the PFN1 WT structure (Top). These contacts are reduced by the M114T mutation (Bottom) owing to the smaller size of threonine, leading to an enlargement of the surface-exposed pocket. (B) PFN1 WT is shown with a transparent surface and the secondary structure is shown in cartoon representation. The surface pocket volume for PFN1 WT (green) and the cleft volume are generated using SiteMap. The predicted cavity (blue) for PFN1 C71G (generated using PyMOL) overlays with the M114T void, and unlike the WT and M114T volumes, is not surface-exposed. The insets (Right) show the aforementioned voids for WT (Top), M114T chain B (Middle), and C71G (Bottom).
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