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Keywords
amyotrophic lateral sclerosis, profilin 1, protein stability, X-ray crystallography, protein misfolding

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

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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.

This article is a PNAS Direct Submission.

Data deposition: The crystallography, atomic coordinates, and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 4X1M, 4X1M, and 4X25).

1S.B. and T.V.S. contributed equally to this work.

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Editing by Gregory A. Petsko, Weill Cornell Medical College, New York, NY, and approved May 14, 2015 (received for review December 16, 2014)

Mutations in profilin 1 gene (PFN1) were recently associated with both familial and sporadic forms of amyotrophic lateral sclerosis (ALS) (1, 2), an incurable and fatal neurodegenerative disease that primarily targets motor neurons (3). The etiology of sporadic ALS is poorly understood, whereas familial ALS is caused by inheritable genetic defects in defined genes such as PFN1 (3). PFN1 is a 15-kDa protein that is best known for its role in actin dynamics in the context of endocytosis, membrane trafficking, cell motility, and neuronal growth and differentiation (4). In addition to binding monomeric or G-actin, PFN1 also binds to a host of different proteins through their poly-L-proline motifs and to lipids such as phosphatidylinositol 4,5-bisphosphate (4, 5). However, little is known about the mechanism(s) associated with PFN1-mediated ALS pathogenesis. The observation that most ALS-linked PFN1 variants are highly prone to aggregation in mammalian cultured cells suggests that disease-causing mutations induce an altered, or misfolded, conformation 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.

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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.

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

| Variant | ΔG°, kcal·mol⁻¹ | m, kcal·mol⁻¹·M⁻¹ | Cm, M | Tm, °C | Protein alone | + 4 mM proline | Kd, μM | Binding to poly-γ-proline¹⁻¹³
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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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<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>
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<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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*Errors are shown as SD.
†Errors are shown as SE.
‡Kd values are reported in terms of proline residues.

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 mutations 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 suggested these proteins adopt similar tertiary structures as well (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 predominately 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 resoluibilized 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).

Residues 22–36, 46–52, 101–105, 112–120, and 125–128 within PFN1 were used for Co superimposition of the four molecules (PFN1 WT, M114T chains A and B, and E117G). In agreement with the biochemical analyses described above (Table 1 and Fig. S4), the secondary and tertiary structures of all three PFN1 proteins, including chains A and B of M114T, are highly similar (Fig. 3). Although the space groups for PFN1 WT and M114T crystals were different, we calculated the double difference plots between these and the other PFN1 structures to get a sense for structural perturbations potentially induced by the ALS-linked mutations. Double difference plots were constructed by calculating the distances between all of the Ca atoms in PFN1 WT and an ALS-linked variant separately, and then plotting the difference of the difference between PFN1 structures as described previously (23). Virtually no structural deviations were observed between PFN1 WT and E117G, whereas moderate differences were detected between WT and M114T (Fig. S6).

Next we sought to determine whether these moderate structural changes between PFN1 WT and M114T mapped to regions involved in PFN1 function, namely to residues that make contact with actin (24–31) or poly-l-proline (21, 22, 24, 32, 33). The ternary complex comprised of PFN1 WT, actin, and the poly-l-proline peptide derived from vasodilator-stimulated phosphoprotein (VASP) (21) (PDB ID code 2PAV) is shown in Fig. 4. Residues with the highest (0.3 A or greater) average of absolute double difference (Avg-Abs-DD) values between PFN1 WT and M114T chain B (Fig. S6C) were mapped onto PFN1 WT (Fig. S7). PFN1 M114T chain B was used for this and all subsequent structural comparisons because chain B had lower B factors compared with chain A (Fig. S8). Indeed, several PFN1 residues that reportedly make contacts with actin (V119, H120, G122, and K126) and poly-l-proline (W4, Y7, H134, and S138) also have relatively high Avg-Abs-DD values (Fig. S7).

To assess whether these mutation-induced structural changes are sufficient to alter the normal binding interactions of PFN1,
we first monitored changes in the intrinsic tryptophan fluorescence of PFN1 as a function of poly-L-proline peptide concentration (Fig. 5A). Our results revealed that the effect of ALS-linked mutations on the PFN1-poly-L-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-L-proline effectively stabilized all PFN1 proteins as determined by DSF, with the largest increase in $T_m$ observed upon poly-L-proline 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 pyrenyliodoacetamide-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-L-proline or actin.

Importantly, the X-ray crystal structures reveal a possible mechanism by which ALS-linked mutations destabilize PFN1. Residues Thr90, Met114, and Gln18 contribute to the formation of a surface exposed pocket that was detected using SiteMap (Fig. 7). Mutation of methionine to threonine at position 71 creates an internal cavity that is predicted to partially overlap the cleft formed by M114T, raising the intriguing possibility that the C71G mutation on the PFN1 M114T crystal structure (Fig. 7B). Analysis using PyMOL and SiteMap suggest that, unlike the solvent-accessible WT and the M114T pocket, the proposed C71G void is buried within the core of the protein. Solvent-inaccessible voids have a more destabilizing effect than solvent-exposed cavities (11, 35), providing an explanation for why the C71G mutation is more destabilizing than M114T (Fig. 1).

Discussion

Here we show that ALS-linked mutations severely destabilize (Fig. 1) and alter the native protein conformation (Fig. 3) of PFN1. Changes in protein stability owing to disease-causing mutations, whether these mutations stabilize or destabilize the protein, are thought to play a pivotal role in various disease mechanisms (13). In the context of ALS, disease-linked mutations destabilize Cu,Zn-superoxide dismutase (SOD1) (9), but instead hyperstabilize TAR DNA-binding protein 43 (TDP-43) (8, 10, 36). These findings underscore the importance of defining toxic properties of disease-linked proteins, thereby directing the rational design of therapeutic strategies against those offending proteins (3).

Our X-ray crystal structures of PFN1 proteins illuminate a probable source of mutation-induced destabilization. An enlarged surface pocket, or void, forms as a result of the M114T mutation (Fig. 7). The destabilizing effect of similar voids has been demonstrated using a systematic site-directed mutagenesis approach with lysozyme and is thought to arise from a loss of hydrophobic interactions (11, 35). Examples of mutation-induced cavity formation and destabilization have also been observed in nature (13). Interestingly, modeling the removal of the cysteine side chain at position 71 creates an internal cavity that is predicted to partially overlap the cleft formed by M114T, raising the intriguing possibility that both mutations destabilize PFN1 through a common mechanism that involves the loss of hydrophobic and van der Waals contacts within the same region of PFN1 (Fig. 7). Because

![Image](image-url)
G118V 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 culminates 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.

Materials and Methods

A pET vector containing human PFN1 flanked by Ndel and EcoRI restriction sites was kindly provided by Bruce Goode, Brandeis University, Waltham, MA. The mutant PFN1 DNA (2) was amplified using primers 5′-GGACCATATGCGCCGGTGGAAC-3′ and 5′-GCGTGAATCTCTAGACGGCGGCA-3′ and ligated into the pET vector using NdeI and EcoRI restriction sites. BL21 (DE3) pLysS cells (200132; Agilent Technologies) transformed with PFN1 constructs were cultured in LB containing 100 μg mL⁻¹ ampicillin and 34 μg mL⁻¹ chloramphenicol at 37 °C until an OD₆₀₀ of 0.7, at which point PFN1 expression was induced by addition of 1 mM isopropyl β-D-thiogalactopyranoside (0487; Amresco) for either 3 h at 37 °C (for WT and E117G) or 24 h at 18 °C (for C71G, M114T, and G118V). Cells were harvested by centrifugation and stored until purification. Refer to Supporting Information for complete details on methods.

![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.](https://www.pnas.org/doi/10.1073/pnas.1424108112)
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