Conformational Stability and Pathogenic Misfolding of the Integral Membrane Protein PMP22

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Supplementary Figure 1. Near-UV CD spectra of folded and unfolded PMP22 Variants. WT and mutant PMP22s were equilibrated in non-denaturing (0.00 XLS) and denaturing (0.50 XLS) micelles as described in Materials and Methods prior to acquisition of three replicate near-UV spectra for each variant under each condition. A) The average mean residue ellipticity from three replicate spectra of WT PMP22 at 0.00 XLS (cyan) and at 0.00 XLS (red) is plotted against the wavelength. The average mean residue ellipticity from three replicate spectra of B) H12Q, C) L16P, D) S22F, E) A67T, F) M69K, G) S76I, H) G93R, I) G107V, J) T118M, K) I137V, and L) G150D PMP22 at 0.00 XLS (blue) and at 0.00 XLS (red) is plotted against the wavelength along with the average mean residue ellipticity from three replicate spectra of WT PMP22 at 0.00 XLS (black), which is shown for reference.
Supplementary Figure 2. Effect of the S22F mutation on Zn$^{++}$ binding. S22F PMP22 was titrated with ZnCl$_2$, as described in Materials and Methods, and binding was monitored by the change in the intensity of tryptophan fluorescence emission intensity. The relative fluorescence intensity at 345 nm from a representative replicate experiment is plotted against the concentration of ZnCl$_2$ (red) and the global fit line for two-site binding is shown in red. A representative replicate experiment for WT binding is shown for comparison (black).
Supplementary Figure 3. Influence of pH on the Structural Properties of G107V. WT (Black), S22F (Cyan), and G107V (Red) PMP22 were equilibrated in either 25 mM sodium acetate (pH 5.5) containing 150 mM NaCl, 1.0 mM TCEP, and 28 mM DPC or 25 mM HEPES (pH 7.5) containing 150 mM NaCl, 1.0 mM TCEP, and 28 mM DPC prior to acquisition of their tryptophan fluorescence emission spectra under each condition. The relative fluorescence emission intensity is plotted against the wavelength for each variant at A) pH 5.5 and at B) pH 7.5. All three variants have similar spectra at pH 5.5 (the condition used herein), which suggests that these variants have similar tertiary structures under this condition. The tryptophan fluorescence spectra of WT and S22F PMP22 were observed to be insensitive to variations in pH from 5.5-7.5. However, a significant decrease in tryptophan fluorescence is apparent for G107V near physiological pH, which suggests the effects of this mutation on the tertiary structure of PMP22 are pH-dependent.
Supplementary Figure 4. Cellular trafficking and conformational stability of the apoform of PMP22 variants *in vitro*. The average relative immunostaining of PMP22 variants at the plasma membrane of MDCK cells, as determined from three replicate flow cytometry trials, is plotted against the corresponding change in the free energy of folding determined by near-UV CD as detailed in Materials and Methods. Error bars reflect the standard deviation of the relative immunostaining and the propagat error from $\Delta \Delta G_{F-U}$ calculations.
Supplemental Theory

**Influence of Zn$^{2+}$ binding on the conformational equilibrium and cellular trafficking of PMP22.**

We show in Figure 3B that the binding of Zn$^{2+}$ ions is thermodynamically coupled to the conformational equilibrium of PMP22. In order to elucidate the physical origins of PMP22 misfolding, we sought to assess the impacts of these mutations on PMP22 folding and on the binding energetics. However, conventional experimental approaches to compare the influence of pathogenic mutations on these two processes are undermined by the precipitation of denaturing detergents in the presence of Zn$^{2+}$. Therefore, we came up with a simplified approach to correct apparent binding energetics of mutants variants for differences in the stability of the apoprotein, as follows.

In order to minimize the potential for over-fitting the data, we first opted to fit the data with a two-site binding model and then correct the apparent equilibrium dissociation constants for the differences in the stability of the apoprotein. To do so, we derived an equation that relates the apparent binding affinity ($K_{d,app}$) to the true binding affinity ($K_d$) and the equilibrium constant for the unfolding reaction ($K_u$). Assuming the unfolded state does not bind Zn$^{2+}$ ions under these experimental conditions, this can be accomplished with the following simplified definition of the apparent equilibrium dissociation constant for the cooperative binding of two metal ions:

$$K_{d,app} = \frac{[L]^2 ([N]+[U])}{[N \cdot 2L]} \quad (1),$$

where $[L]$ is the concentration of Zn$^{2+}$, $[U]$ is the concentration of unfolded protein, $[N]$ is the concentration of the folded apoprotein, and $[N \cdot 2L]$ is the concentration of the doubly bound protein. The true equilibrium constants for folding and dissociation can be expressed as follows:

$$K_u = \frac{[U]}{[N]} \quad (2)$$

$$K_d = \frac{[N] [L]^2}{[N \cdot 2L]} \quad (3)$$

By combining equations 1 and 2, the following relationship between $K_{d,app}$ and $K_u$ emerges:

$$K_{d,app} = \frac{[N] [L]^2 (1+K_u)}{[N \cdot 2L]} \quad (4)$$

By combining equations 3 and 4, the following relationship between $K_{d,app}$, $K_u$, and $K_d$ emerges:

$$K_{d,app} = K_d (1 + K_u) \quad (5)$$

The $K_{d,app}$ for the dissociation of two metal ions can be calculated from the experimentally determined equilibrium dissociation constants for the sequential binding and folding reactions as follows:

$$K_{d,app} = K_{d1,app} K_{d2,app} \quad (6)$$

By combining equations 5 and 6, we get the relationship between the experimentally derived $K_{d1,app}$, $K_{d2,app}$, $K_u$, and the true binding affinity $K_d$ as follows:

$$K_{d1,app} K_{d2,app} = K_d (1 + K_u) \quad (7)$$

Equation 7 allows us to calculate the true $K_d$ from the experimentally determined $K_{d,app}$ and $K_u$ values. These corrected $K_d$ values allow us to determine the effect of each mutation on the free energy of binding ($\Delta \Delta G_{bind}$).
Comparison of $\Delta \Delta G_{F-U}$ (effects of mutations on apoprotein folding, Table 1) and $\Delta \Delta G_{\text{bind}}$ values to the relative degree of plasma membrane trafficking may provide insights as to whether the energetic effects of mutations on binding or folding alone can account for the observed differences in the trafficking patterns. Unfortunately, four of the variants are completely unfolded at equilibrium, which prevents accurate determination of either $\Delta \Delta G_{F-U}$ or $\Delta \Delta G_{\text{bind}}$. Furthermore, we must also exclude G107V (pH dependent defects) and S22F (structural changes in the bound state) from the analysis. A plot of the relative plasma membrane trafficking versus the corrected $\Delta \Delta G$ values for folding and for binding for the remaining six variants are shown below.

These plots reveal no obvious relationship between the effects of mutations on binding or folding and the extent of mistrafficking in the absence of an effect of Zn$^{2+}$. These plots along with Figure 5A suggest that it is the combined effects of mutations on both binding and folding that dictate the extent of cellular PMP22 misfolding. To further explore this possibility, we utilized the corrected thermodynamic parameters to simulate the influence of Zn$^{2+}$ binding on the effective equilibrium constant for folding ($K_{\text{fold, app}}$) using the following relationships (adapted from Park & Marqusee, see Supplementary Reference 1):

$$K_{\text{fold, app}} = \frac{[N]+[N+2L]}{[U]} = \frac{(1+\frac{[N+2L]}{[N]})}{K_U} = K_F (1 + \frac{[L]^2}{K_d}) \quad (8),$$

where $K_F$ is the equilibrium constant for the folding reaction (inverse of $K_U$). Based on simulations utilizing equation 8, we find that a linear relationship between the export efficiency and $K_{\text{fold}}$ emerges above Zn$^{2+}$ concentrations of about 700 µM ($R^2 = 0.63$ at 700 µM), as is illustrated in the following plots:

This trend is fully consistent with the expected relationship between the folding energetics and the trafficking efficiency of soluble proteins in the secretory pathway as has been previously predicted by the FoldEx model (see Reference 26). While zinc is known to be abundant in myelin (see References 28 and 29), the
precise concentration of Zn\textsuperscript{2+} ion in the relevant cellular compartments is unclear. Moreover, the concentration of Zn\textsuperscript{2+} required for saturation of PMP22 is likely to be much lower in natural membranes at physiological pH. For these reasons, the quantitative predictions of this simulation are not to be taken at face value. Nevertheless, the conclusion of this exercise confirms that the influence of Zn\textsuperscript{2+} binding on the conformational equilibrium represents a critical energetic factor governing the folding and misfolding of PMP22 in the cell.
Supplementary Table 1. Motor Nerve Conduction Velocity Measurements from Patients Carrying Heterozygous PMP22 Mutations

| Variant | Disease | Number of Patients | Number of Recordings | Average NCV (m s\(^{-1}\)) | Supplementary Reference |
|---------|---------|--------------------|----------------------|-----------------------------|-------------------------|
| S22F    | HNPP    | 5                  | 10                   | 27 ± 4                      | 2                       |
| A67T    | HNPP    | 1                  | 14                   | 40 ± 8                      | 4                       |
| G93R    | CMT1    | 1                  | 2                    | 17                          | 5                       |
| T118M   | CMT1    | 3                  | 7                    | 29 ± 19*                    | 3 6                     |
| M69K    | DSS     | 1                  | 1                    | 3.3                         | 7                       |
| L16P    | DSS     | 7                  | 7                    | 11 ± 8                      | 8                       |
| G150D   | DSS     | 1                  | 1                    | 2.5 ± 2.5**                 | 9                       |
| S76I    | DSS     | 2                  | 2                    | 1.5                         | 10 3                    |
| H12Q    | DSS     | 1                  | 1                    | 7                           | 11                      |
| G107V   | CMT1    | 17                 | 17                   | 23 ± 9                      | 12                      |

Motor nerve conduction velocity (NCV) measurements from the median nerve, ulnar nerve, and peroneal nerve were compiled from the literature. In cases where multiple recordings have been reported, the average values of the available measurements were used to represent the NCV ± standard deviation. NCV values were scored as 0 m s\(^{-1}\) in cases where the NCV was reported as absent. An NCV value of 55 ± 5 m s\(^{-1}\) was assumed for healthy individuals carrying two WT PMP22 genes. Because individuals carrying the I137V mutation are asymptomatic, the NCV value was also represented as 55 ± 5 m s\(^{-1}\).

* Roa et al. report NCV recordings from multiple genotypes. For the sake of consistency, only the NCV value from the patient carrying one copy of WT and one copy of T118M PMP22 was used from this reference.

** Ionasescu et al. report an NCV of < 5 m s\(^{-1}\). For the sake of simplicity, we have represented this value as 2.5 ± 2.5 m s\(^{-1}\), which reflects the range of possible NCV values for this patient.
Supplementary Table 2. Apparent Zn(II) Binding Kinetics of WT and Mutant PMP22s

| Variant | $t_{1/2, \text{obs}}$ (min) |
|---------|-----------------|
| WT      | 0.86 ± 0.03     |
| H12Q    | 1.54 ± 0.04     |
| L16P    | 0.61 ± 0.04     |
| S22F    | 0.30 ± 0.01     |
| A67T    | 0.32 ± 0.02     |
| M69K    | 1.40 ± 0.08     |
| S76I    | 1.04 ± 0.04     |
| G93R    | 0.48 ± 0.07     |
| G107V   | 1.28 ± 0.04     |
| T118M   | 0.57 ± 0.01     |
| I137V   | 0.24 ± 0.01     |
| G150D   | 0.38 ± 0.06     |

Zn(II) binding kinetics were assessed by monitoring the increase in tryptophan fluorescence intensity at 345 nm over time upon the addition of ZnCl$_2$. Rate constants were determined by fitting the observable phase with an exponential function, which contained a linear correction for photobleaching when appropriate. Observed binding kinetics were similar throughout the experimental range of [ZnCl$_2$] for WT PMP22 (data not shown), which suggests refolding of the protein is the rate limiting process for the binding reaction. The half-lives for binding reported above were measured upon the addition of 80 mM ZnCl$_2$ for the mutants and 87 mM ZnCl$_2$ for WT and the error values reflect the standard error from curve fitting.
Supplementary References

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