Control of human VDAC-2 scaffold dynamics by interfacial tryptophans is position specific

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Membrane proteins employ specific distribution patterns of amino acids in their tertiary structure for adaptation to their unique bilayer environment. The solvent-bilayer interface, in particular, displays the characteristic ‘aromatic belt’ that defines the transmembrane region of the protein, and satisfies the amphipathic interfacial environment. Tryptophan—the key residue of this aromatic belt—is known to influence the folding efficiency and stability of a large number of well-studied α-helical and β-barrel membrane proteins. Here, we have used functional and biophysical techniques coupled with simulations, to decipher the contribution of strategically placed four intrinsic tryptophans of the human outer mitochondrial membrane protein, voltage-dependent anion channel isoform-2 (VDAC-2). We show that tryptophans help in maintaining the structural and functional integrity of folded hVDAC-2 barrel in micellar environments. The voltage gating characteristics of hVDAC-2 are affected upon mutation of tryptophans at positions 75, 86 and 221. We observe that Trp-160 and Trp-221 play a crucial role in the folding pathway of the barrel, and once folded, Trp-221 helps stabilize the folded protein in concert with Trp-75 and Trp-160. We further demonstrate that substituting Trp-86 with phenylalanine leads to the formation of a stable barrel. We find that the region comprising strand 44 (Trp-86) and 510-14 (Trp-160 and Trp-221) display slower and faster folding kinetics, respectively, providing insight into a possible directional folding of hVDAC-2 from the C-terminus to N-terminus. Our results show that residue selection in a protein during evolution is a balancing compromise between optimum stability, function, and regulating protein turnover inside the cell.

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1. Introduction

Integral membrane proteins require appropriate positioning of amino acids that help in anchoring the folded scaffold to the amphipathic interface of the bilayer membrane. They require hydrophilic residues in the loops and solvent-exposed extramembrane segments to interact with the aqueous environment and lipid head groups, while hydrophobic residues accommodate themselves towards the tail region of lipids. Tryptophan, tyrosine and lysine are usually found in the region at the interface of the lipid molecules and aqueous environment. These residues help in docking the transmembrane segment of the protein within the membrane due to their ability of ‘snorkeling’ at the interface [1,2]. Tryptophans and tyrosines often form an ‘aromatic belt’ in membrane proteins that stabilize the native structure of the protein, and contribute immensely to the overall protein stability [2–4]. Considering the high cost of tryptophan synthesis in the cell, particularly in lower organisms [5], the indole moiety is incorporated judiciously in proteins. Hence, the enrichment of tryptophans in membrane proteins [6] illustrates the importance of this aromatic residue for the overall protein scaffold formation and its stability within membranes.

Tryptophan is not only the most polarizable residue, but also has the capability to form a repertoire of interactions including hydrogen bonds, π-stacking, hydrophobic interactions, N—H...π and C—H...π [7]. The diverse interaction network involving tryptophan allows this residue to serve as a strong anchoring point for membrane proteins. Recent experimental evidence points to a contextual contribution of the indole ring to the overall membrane protein stability [8]. In less polar environments, the contribution of tryptophan is highest at the lipid hydrophobic core, while in environments that resemble cellular conditions, the...
greatest stabilizing contribution for tryptophan was seen at the interface [8]. An overall stabilizing effect of tryptophans is, however, anticipated, depending upon its positioning according to membrane depth [9]. Furthermore, previous studies on poly-leucine helix forming peptides have suggested that while phenylalanine is energetically stabilized towards the hydrophobic lipid core [10], tryptophan pulls the peptide to the lipid-water interface [1].

Extensive analysis using the transmembrane β-barrel model protein OmpA has revealed that the ‘driving force for folding and stability’ is conferred by the interface aromatics [11]. Such stabilizing effects are magnified by the formation of aromatic clusters. This is facilitated by a preferential localization of the phenylalanine side chain towards the lipid core, with positioning of tryptophan and tyrosine towards the interface [8]. Free energy values derived for the aromatic residues using other transmembrane barrels such as OmpA and Ail establish an important role for interface tryptophans to the folding of β-barrels as well as the post-folding structural stabilization [12,13]. Indeed, in the case of the bacterial outer membrane β-barrel PagP, a solvent-exposed tryptophan located in the N-terminal helix was found to be critical for anchoring the folded barrel to the membrane [14,15].

The thermodynamic and folding studies of bacterial outer membrane proteins have helped us understand how interfacial tryptophans stabilize transmembrane β-barrel proteins [8,11–13]. As mitochondria possess a bacterial ancestry, one may presume that β-barrels of the mitochondrial outer membrane, such as the translocases, sorting and assembly machinery, and porins also possess interfacial tryptophans that confer a stabilizing role to the protein scaffold. However, experimental evidence in this direction is still lacking. In this study, we address the importance of interfacial tryptophans of the human voltage-dependent anion channel isoform 2 (hVDAC-2). hVDAC-2 is one of the porins found in the mitochondrial outer membrane of eutherian mammals, and is involved in maintaining metabolite flux [16]. Using biophysical techniques, we have examined the contribution of tryptophans and consequence of its substitutions on the folding, stability, and function of this outer mitochondrial membrane (OMM) transmembrane β-barrel protein.

hVDAC-2 belongs to the family of primary channel transporters in the OMM. While it is involved in maintenance of cellular homeostasis and transport of metabolites across the OMM [17], hVDAC-2 mainly contributes to cell survival by binding and inhibiting the Bcl-2 family protein BAK [18]. It is a 19-stranded asymmetric barrel with an N-terminal solvent-exposed helix that docks within the barrel, and is important for voltage gating [19]. While we have a tentative mechanism for hVDAC-1 folding [20], experimental evidences for hVDAC-2 folding is still lacking. hVDAC-2 has four tryptophan residues, all of which are strategically placed at the lipid-water interface, on opposite faces of the barrel (Fig. 1). To study the contribution of these tryptophans to barrel stability and function, we constructed mutants with single tryptophans (W86,160,221F, W75,160,221F, W75,86,221F and W75,86,160F) and single tryptophan substitution mutants (W75F, W86F, W160F, W221F) (Table 1).

Membrane protein interfaces are highly sensitive to mutagenesis [11], as they directly affect the correct positioning of the transmembrane segment in lipid membranes. Hence, we substituted tryptophan with phenylalanine, as the latter provides a similar aromatic nature and preferentially interacts with the hydrophobic region of the lipid. Using fluorescence measurements, we show that tryptophan contribution to the hVDAC-2 barrel stability is position dependent, as seen for other bacterial β-barrels [11,13]. We find that tryptophans at two positions, namely, 86 and 160, when substituted with phenylalanine, increase the stability of folded hVDAC-2 barrel in detergent micelles. The other two positions can accommodate both Trp and Phe, as the barrel stability remains similar in both cases. However, we find that all Trp → Phe substitutions considerably decrease the secondary structure content of hVDAC-2. Further, tryptophans 75, 86 and 221 are important for hVDAC-2 gating. Hence, we hypothesize that evolution has chosen to retain tryptophans at these positions to maintain a balance between hVDAC-2 stability and function within the cell, which are together very important for maintaining cellular homeostasis.

2. Materials and methods

2.1. Preparation of folded hVDAC-2 WT protein and its mutants

Cloning of human VDAC-2 wild type gene was carried out using established protocols [21]. Single and multi-tryptophan mutants were generated using site-directed mutagenesis. The mutants are named according to the position wherein the tryptophan is substituted to phenylalanine (Table 1). Single tryptophan mutants are named as W86,160,221F, W75,160,221F, W75,86,221F and W75,86,160F, corresponding to the presence of single-Trp at 75th, 86th, 160th and 221st positions, respectively. Multi-tryptophan mutants are named as W75F, W86F, W160F, W221F and W75,86,86,221F. For example, W75F has all the tryptophans except at the 75th residue (W75 → F). The mutant where all four tryptophans were replaced with phenylalanine was designated W75,86,160,221F (Trp-less mutant). Studies with this mutant were restricted to global secondary structure analysis and simulations.

All the genes were cloned in pET-3a vector. Escherichia coli BL21 (DE3) chemically competent cells were transformed with these plasmids, for protein production in the form of inclusion bodies. Isolation of inclusion bodies was carried out using reported protocols [22] with minor modifications (2.75% triton X-100 was used in the wash step) and further purified by anion-exchange chromatography using established methods [21].

Fig. 1. Schematic of I-TASSER modeled hVDAC-2 WT barrel showing the side (left panel) and top view (right panel). The barrel (shown as blue cartoon) highlights the positions of the four tryptophans (red sticks) with respect to the bilayer membrane (grey spheres in the left panel). Of the four intrinsic tryptophans, three (Trp-75, Trp-160 and Trp-221) lie close to the interface, while Trp-86 lies towards the interior. The position of the bilayer membrane was defined by superposing the hVDAC-2 structure on the zebrafish VDAC-2 structure (PDB ID: 4BUM) obtained from the PDB database [27,28]. IMS, intermembrane space.
2.3. Measurement of rate of folding of hVDAC-2

The folding rates of all the proteins at low GdnHCl concentrations (<0.8 M) were measured at 4 °C using tryptophan anisotropy. Parameters reported earlier [19] were followed for all measurements. Briefly, a \( \lambda_{\text{ex}} \) of 295 nm and \( \lambda_{\text{em}} \) of 340 nm, each with a slit width of 5 nm, were used for anisotropy measurements. The unfolded protein stock (250 \( \mu \)M) in 6 M GdnHCl was rapidly mixed with 10-fold excess of pre-chilled 19.5 mM DDM in Buffer A and 10 mM DTT. The acquisition was started immediately after the mixing. The instrument dead time was ~10 s. This experiment suffers from the limitation that each data point was recorded after ~15.74 s; hence, the first data point is acquired at ~25 s. The data was normalized to obtain folded fraction (\( f_f \)) using the following equation.

\[
f_f = \frac{r - r_u}{r_u} \]

Here, \( r \) is the fluorescence anisotropy at a given time, \( r_u \) and \( r_f \) are the fluorescence anisotropy of folded and unfolded proteins, respectively.

2.4. CD wavelength scans and thermal denaturation measurements

Far-UV wavelength scans were recorded using circular dichroism (CD) spectropolarimetry to obtain the total secondary structure content of the folded tryptophan mutants. To minimize the contribution of noise to the estimations, an average of molar ellipticity values from 214–216 nm \( (\theta_{214-216}) \) was calculated. This was used to decipher the \( \beta \)-sheet content, and hence the folding efficacy, of the hVDAC-2 mutants. The folded protein was also subjected to thermal denaturation (T-scan) from 4–95 °C, and the loss in secondary structure was monitored using far-UV CD at 215 nm. The various thermal denaturation parameters \( T_m, \Delta H_{\text{app}} \) and \( T_m\text{-start} \) were derived from the T-scan experiments as explained previously [19,23].

2.5. Acrylamide quenching and measurement of fluorescence lifetime of tryptophans

All measurements were carried out using established protocols [19] at 25 °C. The effect of acrylamide quenching was studied using steady-state fluorescence [21] and time-correlated single photon counting (TCSPC) [13]. Briefly, samples were incubated with varying concentrations of acrylamide at 25 °C for 5 min, after which, measurements were carried out. Inner filter correction was applied as described previously [21] for steady-state fluorescence measurements. A \( \lambda_{\text{ex}} \) of 295 nm and \( \lambda_{\text{em}} \) of 340 nm was used for both steady-state fluorescence and TCSPC measurements. The average lifetime (<\( \tau \)>) of tryptophan was obtained by fitting the TCSPC data to a triple exponential function [21].

2.6. Equilibrium (un)folding measurements using tryptophan fluorescence

Equilibrium folding and unfolding experiments were carried out by using GdnHCl as the chemical denaturant, with minor modifications of previous protocols [19]. An excitation wavelength of 280 nm was used to improve the signal-to-noise ratio for the single tryptophan mutants and was monitored using Trp fluorescence intensity. As some of the mutants showed hysteresis, the apparent thermodynamic parameters (free energy, \( \Delta G^0 \), cooperativity of folding or unfolding, \( m \) value; mid-point of denaturation, \( C_m \)) were derived for both the unfolding \( (\Delta G^0, m, C_m) \) and folding \( (\Delta G^0, m, C_m) \) pathways, separately.

2.7. Kinetics measurements using tryptophan fluorescence and chevron plots

The folding arm of the chevron plot was generated by manually mixing 25 \( \mu \)M protein in 19.5 mM DDM, 4 M GdnHCl, 10 mM DTT and Buffer A, into a five-fold excess of varying concentrations of GdnHCl (0.8–1.68 M) prepared in Buffer A. For the unfolding arm, 25 \( \mu \)M of the folded protein stock (generated by the folding protocol mentioned in the first section) was diluted 2.5-fold in Buffer A. The obtained mix was then diluted in a 1:1 ratio using a stopped flow accessory (dead time ~8 ms) into varying concentrations of GdnHCl (1.72–3 M) prepared in Buffer A. The final reaction mix for creating both the arms contained 5 \( \mu \)M protein in 3.9 mM DDM, 2 mM DTT, and different GdnHCl concentrations in Buffer A. All the measurements were carried out by measuring the change in tryptophan fluorescence intensity, using \( \lambda_{\text{ex}} = 295 \) nm and \( \lambda_{\text{em}} = 340 \) nm at 25 °C, and the reactions were monitored until no further change in tryptophan fluorescence intensity was observed. For manual mixing, excitation and emission slit widths were 3 nm and 5 nm, respectively, with data accumulation every 6 s to minimize photobleaching. The dead time for manual mixing was ~10 s. For stopped flow experiments, excitation and emission slit widths were 2 and 10 nm, respectively, with data acquisition every 0.1 s.

The data was fitted either to a single or double exponential function, and the rates \( (k_i \text{ rate from folding kinetics}; k_u \text{ rates from unfolding}) \) were determined.
kinetics) were plotted against the respective GdnHCl concentrations to obtain the chevron plots. The linear zone of both the arms was fitted to the following equations [24].

\[
\ln (k_f) = \ln \left( k_{f,0} \right) + \frac{m_{f,\text{kin}} |D|}{RT}
\]

(2)

\[
\ln (k_u) = \ln \left( k_{u,0} \right) + \frac{m_{u,\text{kin}} |D|}{RT}
\]

(3)

wherein \(k_{f,0}\) and \(k_{u,0}\) are the intercepts and \(m_{f,\text{kin}}\) and \(m_{u,\text{kin}}\) are the slopes of folding and unfolding arm, respectively, \(R\) is the gas constant in kcal/mol, \(T\) is temperature in Kelvin and \(D\) is the denaturant concentration. As the unfolding arm had a non-linear profile, a quadratic Eq. (3) was also used for fitting the unfolding arm wherein \(m_{u,\text{kin}}\) parameter accounts for the non-linearity [24].

\[
\ln (k_u) = \ln \left( k_{u,0} \right) + \frac{m_{u,\text{kin}} |D|}{RT} + m_{u,\text{kin}} |D|^2
\]

(4)

The free energy of unfolding (\(\Delta G_{\text{kin}}^0\)) was calculated using Eq. (5).

\[
\Delta G_{\text{kin}}^0 = -RT \ln \left( \frac{k_{f,0}}{k_{u,0}} \right).
\]

(5)

The Tanford \(\beta\) value (\(\beta_f\)) was calculated as reported previously [25].

\[
\beta_f = \frac{-m_{f,\text{kin}}}{m_{u,\text{kin}} - m_{f,\text{kin}}}
\]

(6)

2.8. Molecular dynamics simulations

I-TASSER [26]—modeled hVDAC-2 structure was used as the input file for all the simulations. The structure was first oriented in accordance to zebrafish VDAC-2 (PDB ID: 4BUM) template from Orientations of Proteins in Membranes (OPM) database [27,28], using PyMOL v1.5.0.5 [29]. The assembled hVDAC-2—micelle structure for simulations was generated using the Micelle Builder tool in the CHARMM-GUI web server [30,31]. The W75,86,160,221F (Trp-less) mutant was generated by mutating all the tryptophan residues to phenylalanine, using the PDB manipulation options in the input generator. The hVDAC-2 barrel was subjected to triangular voltage ramps ranging from –60 mV to +60 mV, we observe marginally steeper voltage dependence for almost all single tryptophan mutants, when compared to the WT (Table 2). hVDAC-2 W86,160,221F shows an additional decrease in the \(V_0\) values at positive voltages (Table 2 and Supplementary Fig. 1). Surprisingly, W75,86,221F shows a subconductance state that is slightly different from other tryptophan mutants (lower left panel of Fig. 2). The tryptophan to phenylalanine substitutions carried out in this study are not expected to drastically alter VDAC-2 functional characteristics when compared with other mutations, which change the charged state or chemical nature of the substituted amino acid [38–40]. Hence, the subtle functional differences we observe in a background of conserved substitutions might be sufficient for interpretation. Our data signifies the importance of tryptophans at 75, 86 and 221 in channel gating. The subconductance state achieved at higher voltages displays higher conductivity values when compared to WT, as the response to the applied voltage for this mutant barrel is weak.

We calculated the \(nF_0V\) value, which is an indication of the difference in the energy of open and closed states (Table 2) [41]. All the mutants show a decrease in \(nF_0V\) value, demonstrating a certain degree of conformation rearrangement upon replacement of tryptophans [41]. We find that although tryptophans may not be important for maintaining the pore diameter as they face the lipids, they do affect the overall energy difference between the open and the closed states of the lipid-inserted barrel.

3.2. Folding proceeds from N- to C-terminus in hVDAC-2 barrel formation

Membrane protein folding and factors affecting this process have previously been studied in lipidic micelles and vesicles. We have previously shown that hVDAC-2 WT exhibits rapid folding rates in detergent micelles, while folding in vesicles is inefficient [21]. Hence, we investigated the effect of tryptophan substitutions on the kinetics of hVDAC-2 barrel formation in dodecyl \(\beta\)-o-maltoside (DDM). Our previous experiments indicate that the overall barrel scaffold is well supported by the oblate DDM micelles [19].

We used tryptophan fluorescence anisotropy to monitor the folding kinetics at 4 °C, as tryptophan fluorescence intensity measurements are too fast to be captured accurately at GdnHCl concentrations < 0.8 M [21]. As the protein folds, the fluorescence anisotropy of tryptophan also increases. This is because the indole ring undergoes a change from a solvent-exposed flexible conformation in the unfolded protein to a buried and rigid state in the folded protein. The W75,160,221F mutant, which only possesses Trp-86, shows the slowest folding rate among all the mutants (~30% slower than WT) (Fig. 3 and Supplementary Fig. 2). This tryptophan lies in \(\beta\)-strand. Similarly, the W86,160,221F
The folding of hVDAC-2 is a multi-step process. We are monitoring the folding process of hVDAC-2 using the change in Trp fluorescence as a reporter of the barrel folding rate. Hence, our fluorescence would indicate whether a specific indole is buried early during the folding process. Accordingly, we find that Trp-160 (W75,86,160F) or Trp-221 (W75,86,160F) show faster folding rates. Therefore, one can assume that strands β10–β14 assemble early during hVDAC-2 folding. Similarly, Trp-75 (W86,160,221F) and Trp-86 (W86,160,221F) show slower folding rates than the WT, suggesting that strands β3–β4 assemble later during folding. The folding rate of the WT protein is an average of the folding rates obtained for the single-Trp mutants. Hence, one possible explanation for the data is that the barrel folding proceeds from the C-term (where Trp-160 and Trp-221 reside) region corresponding to β10–β14 to the N-term (where Trp-86 resides; region corresponding to β4).

As tryptophans play an important role in the barrel folding and stability, we probed the overall final folded state of the mutant barrels by measuring their far-UV CD profiles (ME214–216, ellipticity values at 214–216 nm are plotted in Fig. 3C). The secondary structure content of all the single Trp mutants is lower than the WT protein, with W86,160,221F and W75,160,221F remaining the least structured of all the single Trp mutants.

### Table 2

Voltage gating parameters for hVDAC-2 WT and its single tryptophan mutants.

| hVDAC-2 mutants | Positive voltages | Negative voltages | Single channel conductance [μS] |
|-----------------|-------------------|-------------------|-------------------------------|
|                 | n     | V₀     | nFV₀  | n     | V₀     | nFV₀  |                           |
| WT (5)          | 3.12 ± 0.50 | 28.63 ± 1.92 | 8.62  | 3.07 ± 0.47 | −25.00 ± 2.64 | 7.4  | 2.43 ± 0.58 (6), 3.98 ± 0.50 (12) |
| W86,160,221F (4) | 2.54 ± 0.55 | 19.97 ± 1.00 | 4.90  | 1.89 ± 0.23 | −22.13 ± 2.90 | 4.04 | 2.44 ± 0.43 (5), 3.97 ± 0.63 (10) |
| W75,160,221F (4) | 2.80 ± 0.71 | 24.85 ± 3.37 | 6.72  | 3.13 ± 0.31 | −22.59 ± 2.93 | 6.82 | 2.48 ± 0.26 (6), 3.85 ± 0.39 (10) |
| W75,86,221F (4)  | 2.58 ± 0.20 | 23.64 ± 2.85 | 5.89  | 2.31 ± 0.20 | −26.07 ± 1.86 | 5.80 | 2.82 ± 0.21 (6), 3.91 ± 0.37 (8) |
| W75,86,160F (4)  | 2.24 ± 0.61 | 25.63 ± 2.44 | 5.55  | 2.29 ± 0.25 | −24.08 ± 3.22 | 5.33 | 2.46 ± 0.57 (5), 3.74 ± 0.47 (11) |

Error values represent standard deviation between independent experiments.

- **a**: Values in brackets indicate the number of independent experiments conducted for the voltage ramp studies to derive n and V₀ values.
- **b**: V₀ given in mV.
- **c**: nFV₀ given in kJ mol⁻¹.
- **d**: Numbers in parenthesis indicate the total number of channels considered from at least three independent experiments. Channels are segregated based on their insertion in fully open (<4 nS) or in subconductance (~2 nS) states.
- **e**: Parameters for WT are published in reference [19] and are used here with permission for purpose of comparison.

### Figure 2

G/CV₀ plots for all the four single tryptophan mutants derived from multi-channel membranes. Both W86,160,221F and W75,160,221F show minor variation in the subconductance states at positive voltages. W75,86,221F, on the other hand, shows considerable variation at both the positive and negative voltages, and is least sensitive to increasing voltage. Grey symbols indicate the WT protein and is shown here for purpose of comparison. Error bars are omitted for the sake of clarity. The complete data is shown in Supplementary Fig. 1. Data for hVDAC-2 WT protein has been reproduced from reference [19] with permission.
HVDAC-2 barrel. 

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compromised folding efficiency in DDM micelles. This includes the Gibbs free energy ($\Delta G^\circ$), for both the unfolding and folding pathways from the 24 h data. This includes the Gibbs free energy ($\Delta G^\circ$), for both the unfolding and folding pathways (Supplementary Fig. 4B). Hence, we derived apparent thermodynamic parameters [44], for both the unfolding and folding pathways from the 24 h data. This includes the Gibbs free energy ($\Delta G^\circ$), cooperativity of the unfolding or folding process ($m$ value), and midpoint of chemical denaturation ($C_m$). In general, we observe that for all mutants, the folding pathway is more cooperative (higher $m$ value) and correspondingly, has a higher $\Delta G^\circ$ than the unfolding pathway (compare Fig. 4A and B). This shows that folding of the HVDAC-2 barrel is energetically favorable and under optimal conditions, the equilibrium is shifted towards the folded state. The unfolding process, on the other hand, is less cooperative, which can be due to slower denaturant access to the folded barrel upon GdnHCl addition. We also see protein aggregation in all the mutants at lower GdnHCl concentrations, upon prolonged incubation, as observed earlier in the WT barrel [19]. Hence, the aggregation process at lower GdnHCl concentrations is independent of tryptophan residues.

We see a drastic decrease in protein stability when only tryptophan at the 86th position is present (W75,160,221F, Fig. 4), in both the unfolding and folding pathways. Hence, tryptophans at positions 75, 160 and 221 are important for HVDAC-2 stability. The low free energy for W75,160,221F is mainly due to low $m$ values, as the $C_m$ is similar to the WT for all the single Trp mutants. According to the modeled structure, the 86th position is comparatively more buried than the other three tryptophans, which are at the interface (Fig. 1). We find that while the other positions can accommodate both tryptophan and the more hydrophobic phenylalanine without considerably changing barrel stability, phenylalanine at 86th position increases stability. This is also confirmed by the ~1.7 fold increase in stability of the W86F mutant over the W75,160,221F mutant, wherein, W86F differs from the WT barrel at just the 86th position (Fig. 4). The W75,86,F mutant is also destabilized to a similar extent as the W75,160,221F mutant in the folding process, due to lowered $m$ value. We conclude that a highly cooperative folding process of hVDAC-2 is achieved if at least two tryptophans (Trp-75 and Trp-160/160-Trp-221) are present at opposite faces ($\beta$3 and $\beta$10/$\beta$14) of the barrel.

Fig. 3. Folding rates and secondary structure content of hVDAC-2 WT and various tryptophan mutants. (A) Folding kinetics of hVDAC-2 W75,160,221F and W86F monitored using tryptophan fluorescence anisotropy in 19.5 mM DDM at 4 °C, highlights the difference in the rates between the two mutants. Solid lines indicate fits to a single exponential function. The folding kinetics for all the mutants and the raw anisotropy data is shown in Supplementary Fig. 2. (B) Histograms summarizing the rates obtained from (A), after fitting individual data to a single exponential function. hVDAC-2 W75,160,221F shows the slowest folding rate in DDM micelles. The initial dead time of the experiment was ~25 s, and the initial folding phase could not be accurately captured. Error bars specify standard deviation from three independent experiments. (C) $\Delta G_{214-216}$ shows an average of molar ellipticity ($\text{ME}$) values from 214–216 nm, and is used as an indicator of the secondary structure content in folded hVDAC-2. W75,86,F,160,221F (Trp-less) and all the single tryptophan mutants show a compromised folding efficiency in DDM micelles, and therefore, have lower ME values. Put together, these data highlight the importance of tryptophans for proper folding of the hVDAC-2 barrel.

When three tryptophans are replaced with the more hydrophobic phenylalanine in the WT barrel, we conjecture that the benzyl ring preferentially buries itself, leading to a marginally altered barrel structure. This change, in turn, renders the lone tryptophan inaccessible to acrylamide. Such a behavior for phenylalanine has been reported previously when the 221st position has phenylalanine (W221F mutant), the barrel exhibits increased resistance to chemical solvation (highest $C_m$ value) [33]; whereas, W160F shows an average of molar ellipticity ($\text{ME}$) values for Residue 221 displays interesting features in our experiments. First, W75,86,F,160,F is the only single-Trp mutant to display cooperative protein folding and unfolding, comparable to the WT protein. Secondly, the mutation of Trp-221 (in the W221F mutant) shows a marginally lowered $m$ than the other multi-Trp mutants (Fig. 4). Hence, the 221st Trp contributes to the folding and stability of HVDAC-2. Interestingly, when the 221st position has phenylalanine (W221F mutant), the barrel exhibits increased resistance to chemical solvation (highest $C_m$ value) (Fig. 4). Indeed, this is the only construct to exhibit a considerably different $C_m$. Residue 221 is present near to one of the longest loops of the hVDAC-2 protein. A mutation in this region can have implications on

tryptophan mutants (WT, W75,86,F, W75,F, W86,F, W160,F, and W221F) show similar values for the Stern-Volmer constant ($K_{sv}$) (Supplementary Fig. 3A). Further, the lifetimes decrease upon acrylamide addition (Supplementary Fig. 3B). The single tryptophan mutants show different lifetimes (between 2–3 ns), lowered $K_{sv}$ values (indicating that the indoles have less accessibility to acrylamide as compared to the WT barrel), and different fluorescence quenching profiles (Supplementary Fig. 3). When single tryptophan mutants of hVDAC-2 are probed using acrylamide quenching coupled lifetime measurements, we see that mutant with Trp-75 reaches saturation faster than the other mutants (Supplementary Fig. 3B). This indicates a likely added contribution of a vicinal quencher. The neighboring residue of Trp-75 is Cys-76 (see Fig. 7D), which can act as a potent quencher for tryptophan fluorescence [43].

When three tryptophans are replaced with the more hydrophobic phenylalanine in the WT barrel, we conjecture that the benzyl ring preferentially buries itself, leading to a marginally altered barrel structure. This change, in turn, renders the lone tryptophan inaccessible to acrylamide. Such a behavior for phenylalanine has been reported previously in transmembrane helices [1]. Therefore, a lone tryptophan, particularly at 75th or 86th position, is insufficient to define the barrel interface of hVDAC-2.

3.4. Trp-86 is a key deterrent while Trp-221 is a key determinant of HVDAC-2 barrel stability

The four intrinsic tryptophans of hVDAC-2 are placed strategically on the opposing faces (Fig. 1) of the barrel, to define the barrel interface correctly. To better understand the role of each of these tryptophans in barrel stability we subjected the mutants to equilibrium folding and unfolding measurements in DDM micelles, using GdnHCl as the denaturant. The hVDAC-2 WT barrel shows complete reversibility in its (un/re)folding process (Supplementary Fig. 4A). W160F shows considerable hysteresis under these conditions. This mutant retains the three other tryptophans of HVDAC-2, highlighting that the presence of tryptophan at the 160th position is important to maintain the thermodynamic equilibrium of HVDAC-2.

We observe hysteresis to different extent in most of the mutants. Further, increasing the incubation time for more than 48 h, led to protein aggregation at intermediate GdnHCl concentrations, especially in the folding reactions (Supplementary Fig. 4B). Hence, we derived apparent thermodynamic parameters [44], for both the unfolding and folding pathways from the 24 h data. This includes the Gibbs free energy ($\Delta G^\circ$), cooperativity of the unfolding or folding process ($m$ value), and midpoint of chemical denaturation ($C_m$). In general, we observe that for all mutants, the folding pathway is more cooperative (higher $m$ value) and correspondingly, has a higher $\Delta G^\circ$ than the unfolding pathway (compare Fig. 4A and B). This shows that folding of the HVDAC-2 barrel is energetically favorable and under optimal conditions, the equilibrium is shifted towards the folded state. The unfolding process, on the other hand, is less cooperative, which can be due to slower denaturant access to the folded barrel upon GdnHCl addition. We also see protein aggregation in all the mutants at lower GdnHCl concentrations, upon prolonged incubation, as observed earlier in the WT barrel [19]. Hence, the aggregation process at lower GdnHCl concentrations is independent of tryptophan residues.

We see a drastic decrease in protein stability when only tryptophan at the 86th position is present (W75,160,221F, Fig. 4), in both the unfolding and folding pathways. Hence, tryptophans at positions 75, 160 and 221 are important for HVDAC-2 stability. The low free energy for W75,160,221F is mainly due to low $m$ values, as the $C_m$ is similar to the WT for all the single Trp mutants. According to the modeled structure, the 86th position is comparatively more buried than the other three tryptophans, which are at the interface (Fig. 1). We find that while the other positions can accommodate both tryptophan and the more hydrophobic phenylalanine without considerably changing barrel stability, phenylalanine at 86th position increases stability. This is also confirmed by the ~1.7 fold increase in stability of the W86F mutant over the W75,160,221F mutant, wherein, W86F differs from the WT barrel at just the 86th position (Fig. 4). The W75,86,F mutant is also destabilized to a similar extent as the W75,160,221F mutant in the folding process, due to lowered $m$ value. We conclude that a highly cooperative folding process of hVDAC-2 is achieved if at least two tryptophans (Trp-75 and Trp-160/160-Trp-221) are present at opposite faces ($\beta$3 and $\beta$10/$\beta$14) of the barrel.
the strand registry, thereby influencing the chemical solvation of the barrel. We speculate that barrel folding proceeds from the C- to N-terminus (β14 and β10 fold before β4; see Fig. 1). We can explain the ability of Phe-221 to nucleate folding through initial interaction with detergent molecules when we consider the hydrophobic nature of phenylalanine. This property can drive folding of the W221F barrel at higher GdnHCl concentrations, and account for its high Cm.

Overall, a global reduction in the unfolding free energy across all mutants is due to the lowered unfolding cooperativity (low m values). The destabilizing effect of Trp at the 86th position and favorable contribution from 221st position in hVDAC-2 folding are also conserved in the unfolding pathway.

3.5. β3-4 is inherently unstable during folding of hVDAC-2 barrel in DDM micelles

Most of the tryptophan mutants showed hysteresis in equilibrium experiments. Hence, we validated our deductions using the thermodynamic parameters obtained from chevron plots. It must be noted here that on account of hysteresis, the kinetic traces saturate to an intermediate state for the GdnHCl concentrations lying in the transition zone of our equilibrium experiments (~1.5 M – 2.5 M). We obtained the folding arm by manual mixing, and by monitoring the change in the fluorescence intensity of tryptophan as the barrel folds. The folding kinetics fit well to a double exponential function (Supplementary Figs. 5 and 6). This shows that folding of the hVDAC-2 barrel proceeds through two steps. The initial association of the barrel with the micelle, upon dilution of the denaturant, is likely to be rapid, representing the fast phase. Minor structural rearrangements, especially in the vicinity of the 86th position decide the slow phase. We also observed a marginal rollover in the folding arm at very low GdnHCl concentrations, indicating the likely presence of an intermediate. Additionally, we also knew from our equilibrium measurements that hVDAC-2 barrel has a tendency to aggregate at low GdnHCl concentrations, which can also cause this rollover [25]. Hence, we used only the linear region of the folding arm for our subsequent analysis.

The unfolding arm, on the other hand, is very shallow and had a smooth rollover at ~2.5 M GdnHCl (Supplementary Figs. 5 and 6). Here, the tryptophan fluorescence data obtained at low GdnHCl concentration could be defined well with a single exponential function. As the GdnHCl concentration increases beyond ~2.3 M, the kinetics fit well to double exponential function. We fit the unfolding arm both to a linear function (Supplementary Fig. 5), and with a function that considered the rollover (Supplementary Fig. 6), to derive the thermodynamic parameters. As anticipated, fits to the linear function gave us free energy values (ΔG_{unf}^0) that were marginally lower than the latter (Supplementary Table 1); however, the trend in free energy differences among the mutants was consistent with both methods of data analysis. Overall, the chevron analysis indicates the presence of intermediates in hVDAC-2 folding, and the ΔG_{unf}^0 does not account for the total change in the free energy of the system. Hence, the kinetic free energy is underestimated for hVDAC-2 barrel in DDM micelles in our experiments.

Upon comparing the thermodynamic parameters obtained from chevron analysis, we find that W75,160,221F is the least stable of all mutants, followed closely by the W86,160,221F construct. Our kinetics study highlights the inherent instability of the β3-4 region in the barrel. We derived the Tanford β value, which represents how compact the intermediate state is with reference to the folded and unfolded protein states. The Tanford β values are ~0.5 for all the mutants, indicating that the transition state lies between the native and denatured states [45,46], and is not affected by substitution of tryptophan. Considering all these results, we find that unfolding of WT takes place through an intermediate that is not detected in the equilibrium chemical denaturation experiments. Additional experiments that allow for the capture of the unfolding intermediate are needed to characterize its nature. Further, hVDAC-2 W75,160,221F and W86,160,221F have compromised folding kinetics presumably due to the structural rearrangement of the
midpoint of thermal denaturation (A), the highest cooperativity of unfolding. Note, however, that W86F possesses the highest T-scan experiments shown in Supplementary Fig. 7B. hVDAC-2 W75,86,221F and W75,86,160,221F mutants are among the least thermal stable mutants, while W75,86,221F exhibits unfolding (high T

interfacial scale[47], Kyte & Doolittle scale[48], and transmembrane tendency scale [49]) show that Trp-160 has the highest partition energy, and lies in a zone populated with hydrophobic residues.

We addressed the effect of interfacial hVDAC-2 tryptophans on the global folded structure and stability of the folded barrel in DDM micelles. For this, we subjected the folded protein to thermal denaturation from 4–95 °C and monitored the change using far-UV CD at 215 nm. hVDAC-2 undergoes aggregation upon unfolding [19]. The far-UV CD data provides information for both unfolding and aggregation, which we can obtain from the \( T_m \) and \( T_{m\text{-start}} \) (mid-point and unfolding start temperatures, respectively), and the apparent change in the overall enthalpy (\( \Delta H_{\text{app}} \)), which is also an indication of the overall cooperativity of the reaction [19]. The Trp-less mutant (W75,86,160,221F) is one of the most unstable mutants, with lowest \( T_m \) and \( T_{m\text{-start}} \) (mid-point and unfolding start temperatures, respectively) values. The \( \Delta H_{\text{app}} \) is also lowest for Trp-less mutant (Fig. 5). Hence, tryptophans play an important role in anchoring the folded barrel to the micelles, and its removal causes the barrel to unfold in a less cooperative manner over a large temperature range (Supplementary Fig. 7).

All single and multi-tryptophan mutants show lowered \( T_m \) (Fig. 5A). In line with our findings from chemical denaturation, hVDAC-2 W75,160,221F appears destabilized and shows rapid (cooperative) unfolding (high \( \Delta H_{\text{app}} \); Fig. 5C). We believe that due to the initial destabilization and vulnerable folded state of the barrel, hVDAC-2 W75,160,221F collapses rapidly upon heating. On the other hand, W86F mutant emerges as most resistant to thermal denaturation (highest \( T_m \); Fig. 5A). Here again, we find that stability increases when Trp at the 86th position is replaced with a phenylalanine, owing to the buried nature of this region (see Fig. 1). The folded state of W86,160,221F is also comparable to W75,160,221F (Fig. 3C); however, it is not as destabilized (see stability measurements in Fig. 4), and hence unfolds slowly, showing a lower \( \Delta H_{\text{app}} \) (Fig. 5C).

An unexpected finding from the thermal denaturation experiments came from the W75,86,221F mutant. This mutant shows stability levels (lowest \( T_m \)) comparable to the Trp-less mutant. When the 160th position is substituted for phenylalanine (W160F mutant), thermal stability is regained only to some extent. To better understand the environment of 160th position, we calculated the hydration values for hVDAC-2 WT barrel. Hydration values derived using three scales (Willey-White interfacial scale [47], Kyte & Doolittle scale [48], and transmembrane tendency scale [49]) show that Trp-160 has the highest partition energy, and lies in a zone populated with hydrophobic residues (Fig. 6). Trp-221 also lies in a marginally hydrophobic area, whereas the environments of Trp-75 and Trp-86 are amphipathic.

What we find from our thermal denaturation measurements is that except for residue 86, the positions 75, 160 and 221 have incremental contributions to hVDAC-2 stability. This occurs because none of the mutants can completely recover the \( T_m \) and \( \Delta H_{\text{app}} \) of WT. Of the single-Trp mutants, W75,86,221F (mutant retaining Trp-160) is insufficient to thermally stabilize hVDAC-2, whereas barrel destabilization is considerably offset by the presence of Trp-75. The barrel scaffold can accommodate Phe well at position 160, but not to the extent of the 86th position. Hence, we conclude that positions 75, 160 and 221 contribute in an additive manner to stabilize the folded barrel, with a clear preference for Phe only at position 86.

3.7. Unique environment of each hVDAC-2 tryptophans allow for different contributions

We find, from our experimental data, that each hVDAC-2 indole contributes differentially to the voltage gating, folding pathway and post-folding stability of the 19-stranded asymmetric barrel. To further validate our observations, and gain insight into the hydrophobic environment at each Trp position, we carried out atomistic molecular dynamics simulations of hVDAC-2 WT and Trp-less barrels in DDM micelles. Interestingly, when the first 11 residues are not considered, simulations showed no considerable difference in the root mean square deviation (RMSD) and radius of gyration (\( R_g \)) of the two mutants (Fig. 7A). The residues 1–11 of N-terminal helix are highly dynamic only in the WT barrel (Fig. 7B), thereby affecting its whole protein RMSD and \( R_g \) values (Fig. 7A, left panels). We observe three zones of stable RMSD values in the 100 ns trajectory with each of the zones corresponding to different orientations of N-terminal region (Supplementary Fig. 8). Further, \( R_g \) represents compactness of the molecule, and simulations showed that substitution of tryptophans to phenylalanines did not considerably alter the compactness of transmembrane region. The root mean square fluctuation (RMSF) and solvent accessible surface (SAS) area are also similar for both the barrels (Supplementary Fig. 9) showing that the barrel topology remains largely unchanged after the substitution. Hence, our simulation studies indicate that a W → F substitution in hVDAC-2 lowers the dynamicity of the N-terminal helix residues 1–11. The reason for this observation is presently unclear.

Next, we examined the vicinity of each of the four indole (or the corresponding benzyl ring in the Trp-less barrel) positions for gauging the hydration levels and interaction with DDM molecules, over the course of the simulation. In general, we observe an inverse relationship between the hydration level and DDM molecules at the vicinity of both

![Fig. 5. Summary of parameters derived from thermal denaturation experiments of hVDAC-2. Denaturation of the barrel structure was monitored using far-UV CD in 3.9 mM DDM. \( T_m \) midpoint of thermal denaturation (A), \( T_{m\text{-start}} \) starting temperature of secondary structure collapse upon heating (B), and \( \Delta H_{\text{app}} \) cooperativity of unfolding (C) are derived from the T-scan experiments shown in Supplementary Fig. 7B. hVDAC-2 W75,86,221F and W75,86,160,221F mutants are among the least thermal stable mutants, while W75,86,221F exhibits the highest cooperativity of unfolding. Note, however, that W86F possesses the highest \( T_m \).](image-url)
The dynamic nature of information.

Fig. 6. Normalized hydropathy plots for hVDAC-2 WT and W75,86,160,221F (Trp-less) proteins. Hydropathy plots were made using the Willey-White interfacial scale (dark blue,[47]), Kyte & Doolittle plot (dark green,[48]) and transmembrane tendency scale (purple,[49]) with a window size of 9. Regions corresponding to the β-strands are shaded in grey, and the N-terminal helix (residues 17–29) is indicated as cyan. Solid red drop lines mark positions 75, 86, 160 and 221. Residues in the vicinity of position 160 show the highest partition energy, and also lie in a hydrophobic zone.

Fig. 7. In silico analysis of the tryptophan environment in hVDAC-2 barrel probed using molecular dynamics simulations. (A) Comparison of the hVDAC-2 WT and W75,86,160,221F (Trp-less) barrels in DDM micelles along a 100 ns trajectory generated using the GROMACS simulation package.[32] using root mean square deviation (RMSD; top) and radius of gyration (Rg; bottom). hVDAC-2 WT barrel shows variation in RMSD and Rg throughout the trajectory, which is absent in W75,86,160,221F (left panels). This variation stems from the first 11 residues of the N-terminal helix, which exhibit a highly dynamic nature during the simulation. When these residues are not considered in the analysis (right panels), we see that the RMSD and Rg are invariant for both WT and Trp-less mutant. This suggests that mutating tryptophan to phenylalanine does not alter the stability or the compactness of the barrel. Also see Supplementary Fig. 8 for more information. (B) Sausage representation of WT and Trp-less barrels, showing the dynamic nature of first 11 residues in the WT protein. The structure is color coded based on the B-factor (highest: red; lowest: blue).

Tryptophan (in WT) and phenylalanine (in the Trp-less construct) (Fig. 8A and B). A closer examination of the data reveals that although the 160th position is at the interface, we observe a larger number of DDM molecules in its vicinity. The 160th position is therefore comparable to the vicinity of a fully buried lipid-facing residue such as Leu-270, in its DDM content (Fig. 8A and B and Supplementary Fig. 10). Concomitantly, it is also well-hydrated, reflecting the amphipathic nature of this segment (Fig. 8A and B and Supplementary Fig. 10). Residue 160 lies at the start of β10. Both β9 and β10 are the most hydrophobic strands of the barrel, and are expected to bind DDM molecules (Fig. 6). An amphipathic residue at 160th position may, therefore, be important to facilitate strand reversal at the β9–β10 junction and allow for solvation. However, water accessibility in this region also causes the destabilization of hVDAC-2 W75,86,221F upon thermal denaturation, particularly when the other anchoring indoles are absent.

The regions corresponding to 75 and 221 have almost similar number of DDM and water molecules (Fig. 8A and B and Supplementary Fig. 10), and could account for comparable stabilization by Trp or Phe in these positions. These tryptophans are also surrounded by residues like cysteines and glutamic acid that provide an amphipathic environment at the solvent-membrane interface (Fig. 8D). It is known that aromatic interaction networks at the interface sites in transmembrane β-barrels contribute considerably towards its stability.[11] We find that both Trp-75 and Trp-160 have three aromatic residues within a 5 Å distance, while Trp-221 has one (Fig. 8D). The formation of aromatic interactions is one likely mechanism by which the positions 75, 160 and 221 together stabilize the barrel. In contrast to Trp-75, the 86th position only retains a considerable number of DDM molecules in its vicinity (Fig. 8A and B and Supplementary Fig. 10). Trp-86 has no aromatic residues in its vicinity, again supporting the destabilizing effect of this position.

4. Discussion

The contribution of ‘aromatic belts’ formed by tryptophans and tyrosines to membrane protein stability is considerable, and has been studied in detail.[2–4] Herein, we have tried to decipher the role of the four intrinsic conserved tryptophans of the human OMM protein VDAC-2, towards its stability in detergent micelles. From our biophysical analyses, it is evident that the contribution of these tryptophans is clearly dependent upon the position. While all the tryptophans are at the interface in hVDAC-2, our studies show that each position is unique and poses an intrinsic influence on the barrel stability. At the same time, it is also modulated by the placement of either tryptophan or phenylalanine at the other interface positions. This is summarized in Fig. 9, where the various biophysical parameters derived in this study are compared across the mutants.

The hVDAC-2 barrel structure is asymmetric and very similar to VDAC-1, with a height of ~4 nm, and a concave pore of dimensions ~3.5 nm × ~3.1 nm at the interface.[28,50,51] While all four indoles are at the interface, their contributions can be influenced by the local environment. The 86th position tryptophan is buried as compared to the other three positions, and does not have any neighboring aromatic residues (Fig. 8D). The lone tryptophan at this position is considerably inefficient at supporting barrel folding (Fig. 3) and along with W86,160,221F, is the mutant with the least secondary structure content (Fig. 3C). Additionally, this mutant has the least chemical stability (Fig. 4). When the more hydrophobic phenylalanine is placed at this position the barrel stability increases manifold (Fig. 9) owing to its better adjustment to the hydrophobic lipidic interior. Hence, we find that in the presence of Trp-86, and absence of other three intrinsic tryptophans, the β-4 strand is destabilized. However, Trp-86 shows absolute conservation in most of the known VDAC sequences,[52] indicating a possible functional role for β-4 destabilization that is currently unknown.

A tryptophan at residue 75 is seen in VDAC-1 and -2, but is replaced by aliphatic residues in isoform 3.[52] hVDAC-2 barrel stability is moderately affected when substituted with phenylalanine (see the adjacent
placement of W86,160,221F and W75F in Fig. 9). However, Trp-75 does cause a marginal destabilization of $\beta_3$. Similarly, both Phe and Trp are tolerated at position 221. However, both Trp-160 and Trp-221 are highly conserved across all three VDAC isoforms from various organisms [52]. The importance of these regions is also highlighted by its functional relevance in Bak import, tBid induced apoptosis and interaction with steroidogenic acute regulatory protein [53,54]. The 221st position is close to one of the longest loop of the hVDAC-2 barrel. Along with Trp-160, Trp-221 can drive folding of the hVDAC-2 barrel (Fig. 3). While the W221F mutant shows an unusual increase in the barrel’s resistance to chemical solvation (see Fig. 4), we believe that this is due to the intrinsic hydrophobic nature of phenylalanine. Nevertheless, the 221st position can accommodate either tryptophan or phenylalanine in the folded protein, as observed from the close placement of W75,86,160F and W221F in the color scale (Fig. 9).

The true amphipathic nature of tryptophan plays an important role at the 160th position. Although this position is at the interface, it has a hydrophobic surrounding. The nearby aromatic residues provide additional stability to this part of the barrel by forming aromatic interactions (Fig. 8D). The presence of tryptophan only at this position compromises the barrel thermal stability (Fig. 5). We believe that this phenomenon is due to water molecules gaining easier access into the barrel-micelle junctions upon application of heat, when the amphipathic tryptophan is present. The apparent overall stability of the barrel is increased when phenylalanine is present at this position (Fig. 9), as it is able to better adjust itself in the hydrophobic neighborhood.

Overall, it seems that replacement of tryptophan with a phenylalanine causes no change or increases the overall stability of hVDAC-2 barrel. However, the Trp-less mutant shows decreased folding efficiency and secondary structure content. Trp → Phe mutation also introduces hysteresis in the hVDAC-2 barrel that is otherwise maintained under thermodynamic equilibrium. Evolution has thus retained tryptophans...
at these positions to form a functional and stable barrel in the OMM that is also structurally malleable. It has also not escaped our notice that our study has provided the first insight into the in vitro hVDAC-2 folding mechanism. Our experiments show that the C-terminal segments, namely strands [34 and 310], are likely to fold first. This is also supported by the hydrophobic nature of the strands [39–10 and 315–18 (see Fig. 6), which allows this region of the protein to form strong protein-lipid association. The nucleation of folding at the C-terminal strands can then propagate towards the N-terminal strand segments. The slow lipid association. The nucleation of folding at the C-terminal strands (Fig. 6), which allows this region of the protein to form strong protein-lipid interactions, India, for help with the high performance computing facility. We thank all R.M. lab members for useful discussions. R.M. is a recipient of the Wellcome Trust/DBT Intermediate Fellowship.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbcanem.2016.05.011.

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