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Position—Specific contribution of interface tryptophans on membrane protein energetics

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ABSTRACT

Interface tryptophans are key residues that facilitate the folding and stability of membrane proteins. *Escherichia coli* OmpX possesses two unique interface tryptophans, namely Trp76, which is present at the interface and is solvent-exposed, and Trp140, which is relatively more lipid solvated than Trp76 in symmetric lipid membranes. Here, we address the requirement for tryptophan and the consequences of aromatic amino acid substitutions on the folding and stability of OmpX. Using spectroscopic measurements of OmpX-Trp/Tyr/Phe mutants, we show that the specific mutation W76 → Y allows barrel assembly > 1.5-fold faster than native OmpX, and increases stability by ~0.4 kcal mol^{-1}. In contrast, mutating W140 → F/Y lowers OmpX thermodynamic stability by ~0.4 kcal mol^{-1}, without affecting the folding kinetics. We conclude that the stabilizing effect of tryptophan at the membrane interface can be position—and local environment—specific. We propose that the thermodynamic contributions for interface residues be interpreted with caution.

1. Introduction

Integral β-barrel membrane proteins (OMPs) are major structural determinants of the bacterial outer membrane. Among the array of forces that stabilize a membrane protein in its lipid environment, protein-lipid interactions play a key role in deciding the folded functional form of the mature OMP [1,2]. In turn, strategically placed amino acids in the protein scaffold govern protein-lipid interactions. For example, it is seen that aromatic amino acids cluster largely at the solvent-membrane interface [3], and can contribute significantly to the stability of membrane proteins.

Interface tryptophans play an important role in the folding of several bacterial and mitochondrial OMPs [4–8]. Tryptophan exhibits a membrane depth-dependent contribution in its ability to anchor transmembrane β-barrels. While some studies suggest that tryptophan stabilizes OMPs at the bilayer midplane [9], others show that this contribution is highest at the interface [10]. Further, our study with the model OMP PagP showed that tryptophan is less stabilizing than tyrosine or phenylalanine at the interface [11]. Interestingly, it is the rigid aryl ring, and not the hydrogen bonding, which drives the partitioning of tryptophan to the interface [12]. This observation raises questions on why a metabolically expensive amino acid such as tryptophan is required in an aromatic interface girdle that is otherwise enriched with tyrosines and phenylalanines.

To address the need for tryptophan at the bilayer interface, we carried out a detailed thermodynamic analysis on tyrosine and phenylalanine variants of the *E. coli* OMP, OmpX. OmpX has two tryptophans at positions 76 and 140. Both tryptophans of OmpX are located near the membrane interface (Fig. 1A); the indole side chains point toward the membrane and can establish non-covalent interactions with the lipid headgroup and hydrocarbon tail. In OmpX, Trp76 forms a part of the lower aromatic girdle, which also contains two phenylalanine and four tyrosine residues (Fig. 1A). Trp140 resides in the upper aromatic girdle, which is more enriched with aromatic residues, particularly tyrosine (eight tyrosine and three phenylalanine) residues, when compared to the lower aromatic girdle (Fig. 1A). OMPs usually contain tryptophan and tyrosine in the aromatic girdles, while phenylalanine shows a distributed occupancy between the interface and the midplane [13]. We have previously observed that Trp140 is more shielded from the solvent than Trp76 [6]. In folded OmpX, Trp140 is likely to be located closer to the midplane when compared with Trp76, in symmetrical bilayer membranes.

In this study, we generated OmpX mutants where one or both tryptophans were systematically replaced by tyrosine or phenylalanine

Abbreviations: CD, circular dichroism; C_m, midpoint of chemical denaturation; DMPC, PC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, PG, 1,2-dimyristoyl-*sn*-glycero-3-(1′-rac-glycerol); DPC, *n*-dodecylphoshphocholine; E_{act}, activation energy barrier of protein unfolding; f_U, unfolded fraction; k_F, folding rate; ME_{215}, total protein molar ellipticity at 215 nm in deg cm^2 dmol^{-1}; OMP, outer membrane protein; OmpX, outer membrane protein X; OmpX-WT, WT, wild type OmpX containing tryptophans at positions 76 and 140; ΔG^0_U, equilibrium unfolding free energy; θ_{215}, ellipticity in mdeg, at 215 nm

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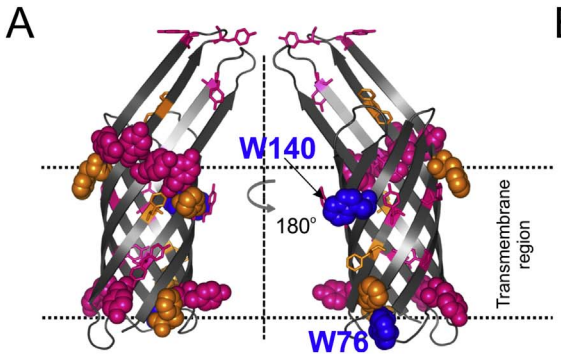

OmpX	Residue	
	76	140
WT	W	W
W76F	F	W
W76Y	Y	W
W140F	W	F
W140Y	W	Y
W76F140F	F	F
W76Y140Y	Y	Y
W76F140Y	F	Y
W76Y140F	Y	F

Fig. 1. OmpX has two interface tryptophans. (A) Ribbon diagram of OmpX barrel highlighting phenylalanines (orange), tyrosines (pink), and the two tryptophans (blue) as spheres (interface) or sticks (others). (B) List of the OmpX mutants discussed in this study.

or both (W76F140Y and W76Y140F) (Fig. 1B). By comparing folding kinetics and equilibrium free energy values, we find that a favorable energetic contribution of an interface tryptophan, when compared to its other aromatic counterparts, can be contextual. Structural plasticity of the extracellular loops of the OmpX family of barrels is required for its binding to host proteins [14]. Hence, we propose that a gain in conformational flexibility at the expense of stability might be required for protein function.

2. Experimental methods

2.1. Protein preparation

OmpX expression, purification, and folding were carried out using reported methods [6,15], and are detailed in the supporting information. The folded protein stock contained 150 μM OmpX, 50 mM DPC (*n*-dodecylphoshphocholine), 50 mM Tris-HCl pH 9.5 and 400 mM urea. Folded protein stocks were diluted 5-fold to 30 μM protein and 10 mM DPC in all experiments, unless specified otherwise.

2.2. Folding rates in micelles and bicelles

The rate of OmpX folding was determined using far-UV circular dichroism (CD) spectropolarimetry on Jasco J815 CD spectropolarimeter (Jasco Inc., Japan). Briefly, 150 μM unfolded protein in 8 M urea was prepared with 50 mM DPC micelles or DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine): DPC bicelles (q = 0.5) at 25 °C. Additionally, folding rates were also determined in DMPC:DPC bicelles doped with 20% DMPG (1,2-dimyristoyl-*sn*-glycero-3-(1′-rac-glycerol)). This corresponds to a DMPC:DMPG ratio of 1:0.25 and an overall bicelle q of 0.5. In the presence of 8 M urea, the protein scaffold is unfolded, but OmpX possesses residual secondary structure [16]. Folding was initiated by the rapid 5-fold dilution of the reaction directly in the cuvette. The final protein concentration was 30 μM and urea concentration was 1.6 M. In micelle samples, the final DPC concentration was 10 mM. In bicelles, the final DMPC and DPC concentrations were 2 mM and 4 mM respectively (q = 0.5). Similarly, 2 mM DPC, 3.2 mM DMPC and 0.8 mM DMPG was used for bicelles of q = 0.5, doped with of 20% DMPG. Heat shock [15] was not used in these experiments. The gain in secondary structure was monitored by measuring the change in ellipticity at 215 nm (θ_{215}), with time. The dead time of the experiment was ~15–20 s. Each data was fitted to a single exponential function to derive the folding rate for the OmpX mutants. The data were then averaged to obtain the mean and standard deviation (s.d.). The end-point samples were also analyzed on cold SDS-PAGE [15] to confirm that each protein was completely folded (data not shown).

2.3. Equilibrium unfolding using circular dichroism

Equilibrium measurements were monitored using far-UV CD. The folded and unfolded protein stocks were diluted 5-fold in various urea concentrations from 80 mM (1.6 M for folding measurements) to 9.5 M at 0.2 M increments, to promote unfolding and folding, respectively. The system attained equilibrium at 216 h, which was confirmed independently using fluorescence measurements. Far-UV CD wavelength scans were recorded between ~205–260 nm at 25 °C [17]; the data were averaged over three accumulations and corrected for buffer and detergent contributions. The value obtained at 215 nm for each urea concentration was normalized between 0 and 1 to obtain the unfolded fraction (f_U) at that urea concentration. Each profile thus obtained was fitted to a two-state linear unfolding model [18,19] provided below:

$$y_O = \frac{(y_F + m_F[D]) + (y_U + m_U[D])\exp[-(\Delta G_U^0 + m[D])/RT]}{1 + \exp[-(\Delta G_U^0 + m[D])/RT]}$$

Here y_O is the observed ellipticity at any denaturant concentration D. y_F and y_U, m_F and m_U are the intercepts and slopes of the folded and unfolded baselines, respectively. ΔG_U^0 is the equilibrium unfolding free energy, m is the change in accessible surface area upon protein unfolding, R is the gas constant (1.987 cal K^{-1} mol^{-1}) and T is the temperature in kelvin. The data were fitted globally using a common m value, and an m value of -1.41 kcal mol^{-1} M^{-1} was obtained from the fit. The thermodynamic parameters (ΔG_U^0 and C_m) were derived for each independent experiment, and then averaged to obtain the mean and s.d. The complete details are provided in the supporting information.

3. Results

3.1. Tyrosine at position 76 of OmpX increases unassisted barrel folding in DPC micelles

The far-UV circular dichroism (CD) spectrum of OmpX is characteristic of a β-sheet rich protein, with a negative trough centered at 215 nm [17]. First, we measured the rate of folding and insertion (k_F) of OmpX, by monitoring the increase in secondary structure content at 215 nm (θ_{215}) (Fig. 2A, first panel). In urea, OmpX possesses residual structure in its denatured state [16,17]. Further, the burst phase [20,21] of OmpX folding falls within the dead time of our experiment (denoted by the arrow in Fig. 2A). Hence, the k_F we obtain represents the gain in β-sheet content as the OmpX β-barrel assembles upon folding [22]. We were able to fit the measured change in θ_{215} to a single exponential function to obtain k_F (the rate of barrel assembly). In *n*-dodecylphosphocholine (DPC) micelles, the k_F of OmpX-WT is ~0.1 min^{-1}. Next, we measured the k_F of the tyrosine and phenylalanine mutants listed in Fig. 1B. The results are shown in Fig. 2B. Surprisingly, we find that introducing tyrosine at position 76 promotes rapid folding of OmpX in DPC micelles. For example, the overall k_F of all OmpX Tyr76 mutants, namely W76Y, W76Y140Y, and W76Y140F, is > 1.5-fold faster in DPC (Figs. 2, S1). To assess if similar rates are obtained in lipidic systems, we measured the k_F for OmpX in DMPC (1, 2-dimyristoyl-sn-glycero-3-phosphocholine): DPC and 20% DMPG doped isotropic bicelles [23,24] of q = 0.5. Here, the k_F of OmpX-WT is