

# Membrane Defects Accelerate Outer Membrane $\beta$ -Barrel Protein Folding

Emily J. Danoff and Karen G. Fleming\*

T. C. Jenkins Department of Biophysics, Johns Hopkins University, Baltimore, Maryland 21218, United States

## S Supporting Information

**ABSTRACT:** Outer membrane  $\beta$ -barrel proteins spontaneously fold into lipid bilayers with rates of folding that are strongly influenced by the physical properties of the membrane. We show that folding is accelerated when the bilayer is at the phase transition temperature, because of the coexistence of lipid phase domains and the high degree of defects present at domain boundaries. These results are consistent with previous observations of faster folding into thin and highly curved membranes, which also contain a higher prevalence of defects. The importance of defects in  $\beta$ -barrel folding provides insight into the intrinsic folding process and the biological assembly pathway.

Transmembrane  $\beta$ -barrel proteins are found in the outer membranes of Gram-negative bacteria, mitochondria, and chloroplasts and are therefore termed outer membrane proteins, or OMPs.<sup>1</sup> These proteins are important for a variety of functions, such as transport of small molecules and proteins, outer membrane maintenance, bacterial virulence, and antibiotic resistance.<sup>1–4</sup> With their myriad of functions and connections to disease, understanding the folding pathway for  $\beta$ -barrel membrane proteins is of significant interest.

In a living cell, OMP folding is facilitated by soluble chaperone proteins<sup>5</sup> as well as the  $\beta$ -barrel assembly machinery (BAM), located in the outer membrane.<sup>6</sup> The BAM complex is built around the protein BamA, which is itself an OMP. Despite the recently determined BamA structures,<sup>7,8</sup> the mechanistic details of how the BAM complex facilitates client OMP incorporation into bacterial outer membranes have not been established.<sup>7,8</sup> *In vitro*, OMPs are capable of spontaneously folding and inserting into lipid bilayers in the absence of other protein factors, which demonstrates the existence of an intrinsic folding mechanism dictated by the amino acid sequence of the protein.

The physical properties of the membrane strongly influence both the rate and the efficiency of OMP folding. Bilayer curvature, hydrophobic thickness, acyl chain saturation, and headgroup composition all affect OMP folding.<sup>9–13</sup> Such membrane modulation of OMP folding rates is biologically relevant, and it was recently suggested that the high kinetic barrier to bacterial OMP folding in the presence of phosphoethanolamine headgroups allows the cell to spatially and temporally control OMP folding.<sup>13</sup> One role of the BAM complex is to overcome this kinetic barrier and facilitate OMP folding into the proper membrane.<sup>13</sup> It should do so by

exploiting those physical properties of the membrane that accelerate OMP folding rates.

*In vitro* folding experiments have shown that OMPs fold faster into membranes with high curvature, such as small unilamellar vesicles (SUVs). Early work revealed that the *Escherichia coli*  $\beta$ -barrel OmpA could spontaneously fold into SUVs composed of the lipids dimyristoylphosphatidylcholine (diC<sub>14</sub>PC) or dioleoylphosphatidylcholine (diC<sub>18:1</sub>PC).<sup>9,10,14</sup> However, under otherwise identical conditions, the level of OMP folding was greatly reduced using lower-curvature large unilamellar vesicles (LUVs) of the same lipids.<sup>11</sup> Similar results have been demonstrated for many other OMPs.<sup>12</sup> The higher folding efficiency in SUVs was attributed to the fact that the strained bilayers of SUVs have a greater prevalence of defects than lower-curvature membranes in LUV geometries.<sup>1,9</sup>

In addition, higher OMP folding efficiencies have been observed in the shorter chain lipids diC<sub>10</sub>PC, diC<sub>11</sub>PC, and diC<sub>12</sub>PC, which form membranes with hydrophobic widths thinner than those of longer chain lipids.<sup>11,12</sup> Moreover, there was a strong correlation between bilayer thickness and apparent folding rate for all OMPs examined; folding was fastest in the thinnest diC<sub>10</sub>PC bilayers, slower in diC<sub>11</sub>PC bilayers, and even slower in diC<sub>12</sub>PC bilayers.<sup>11,12</sup> The accelerated folding in thinner membranes was attributed to an increased prevalence of bilayer defects, consistent with data showing that thinner bilayers are more permeable to solutes<sup>15</sup> and with simulations that demonstrate that thinner bilayers have higher incidences of spontaneous pore formation.<sup>16</sup>

To directly address the idea that membrane defects accelerate  $\beta$ -barrel OMP folding, we conducted folding experiments in LUVs of diC<sub>14</sub>PC at or near the phase transition temperature ( $T_m$ ) of the lipid bilayer, the temperature at which the gel and fluid phases coexist and membranes are known to be more permeable and contain defects.<sup>17–20</sup> Because all other variables were held constant, this strategy allowed examination of the effect on folding caused by bilayer structural changes occurring during the phase transition.

We used differential scanning calorimetry to determine the  $T_m$  for diC<sub>14</sub>PC under our experimental folding conditions and observed a sharp endothermic peak at 24 °C (Figure S1 of the Supporting Information), which is consistent with literature values.<sup>21</sup> We then examined the folding behavior of the well-studied OMP, OmpA<sub>171</sub> (the N-terminal  $\beta$ -barrel domain of OmpA<sup>22</sup>), in diC<sub>14</sub>PC at temperatures below the  $T_m$  (20 °C),

Received: November 21, 2014

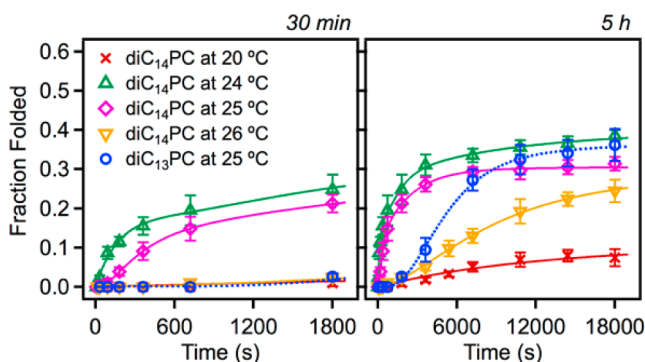
Revised: December 16, 2014

Published: December 16, 2014



at the  $T_m$  (24 °C), and slightly above the  $T_m$  (25 and 26 °C). Folding was monitored using the orthogonal methods of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), which measures tertiary structure formation, and circular dichroism (CD) spectroscopy, which measures secondary structure formation.

Figure 1 shows the folding kinetics measured by SDS–PAGE (see Materials and Methods and Figure S2 of the Supporting



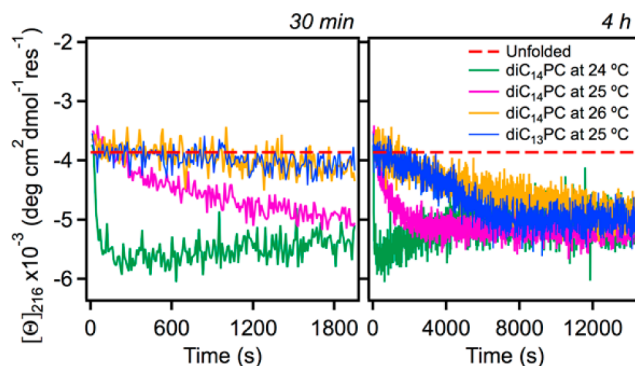
**Figure 1.** Time dependence of the fraction folded measured by SDS–PAGE for OmpA<sub>171</sub> folding into LUVs composed of diC<sub>13</sub>PC or diC<sub>14</sub>PC at 20, 24, 25, or 26 °C. Lines are fits to a quadruple-exponential expression to guide the eye. Data are displayed over two time ranges: 1800 s (30 min) and 18000 s (5 h).

Information). Very little folding was detected in diC<sub>14</sub>PC LUVs at 20 °C (red x), consistent with previous observations that OMPs do not fold into lipid bilayers in the gel phase.<sup>9</sup> The fastest folding was observed at 24 °C (green triangles), with slightly slower folding at 25 °C (pink diamonds) and substantially slower folding at 26 °C (gold upside-down triangles). Therefore, we conclude that OmpA folding is fastest when the bilayer is at the  $T_m$  and folding becomes slower at slightly higher temperatures, as the bilayer becomes uniformly fluid phase.

For comparison, we also measured the folding kinetics in the slightly thinner bilayers of diC<sub>13</sub>PC LUVs, the  $T_m$  of which is 14 °C, which results in fluid phase bilayers at 25 °C.<sup>23</sup> The kinetics in diC<sub>13</sub>PC at 25 °C (blue circles) were markedly slower than in diC<sub>14</sub>PC at 24 and 25 °C. On the basis of the previously established trend of OMP folding being accelerated in thinner bilayers,<sup>11</sup> it would be expected that folding in diC<sub>13</sub>PC would be faster than in diC<sub>14</sub>PC. However, being at or just above the  $T_m$  causes the acceleration of the kinetics in diC<sub>14</sub>PC, resulting in faster folding than in diC<sub>13</sub>PC. The kinetics in diC<sub>14</sub>PC at 26 °C were slower than in diC<sub>13</sub>PC, indicating that in uniformly fluid phase bilayers, the kinetics follow the expected trend.

Figure 2 shows the CD signal change at 216 nm during folding, which indicates  $\beta$ -sheet secondary structure formation (see Figure S3 of the Supporting Information for wavelength spectra). Consistent with the SDS–PAGE data, the kinetics were fastest in diC<sub>14</sub>PC at 24 °C and slowest at 26 °C. Folding in diC<sub>13</sub>PC at 25 °C was also slower than in diC<sub>14</sub>PC at 24 and 25 °C but slightly faster than in diC<sub>14</sub>PC at 26 °C.

The SDS–PAGE and CD kinetic data exhibit multiphasic behavior, indicating a complex folding mechanism. A lag phase becomes apparent under the slower folding conditions; this typically indicates a multistep reaction. In addition, an intermediate conformation is populated in diC<sub>14</sub>PC at 24 °C,



**Figure 2.** CD signal kinetics measured at 216 nm for OmpA<sub>171</sub> folding into LUVs composed of diC<sub>13</sub>PC or diC<sub>14</sub>PC at 20, 24, 25, or 26 °C. The signal for unfolded OmpA<sub>171</sub> at 216 nm is plotted as a red dashed line. Data are displayed over two time ranges: 1800 s (30 min) and 14400 s (4 h).

as evidenced by the intensity of the CD signal decreasing to a more negative value before increasing to the final signal corresponding to the native amount of  $\beta$ -sheet structure. The other CD curves decrease directly to the final value, but this does not necessarily mean that the same intermediate is not populated under those conditions as well. It is likely that the accelerated kinetics at 24 °C result in more rapid formation of the intermediate, allowing it to build up and influence the observed signal. At the higher temperatures and in diC<sub>13</sub>PC, the intermediate may be formed slowly enough that it is subsequently converted to the next conformation before it can build up enough to impact the CD signal. This slowed reaction step could also be inducing the lag phase in the SDS–PAGE data. Further investigation into the conformation of the proposed folding intermediate and the details of the OmpA  $\beta$ -barrel folding pathway will be reported in a separate manuscript.

We propose that the acceleration of OmpA  $\beta$ -barrel folding measured by SDS–PAGE and CD at the transition temperature for diC<sub>14</sub>PC is due to a higher incidence of bilayer defects occurring during the transformation between phases. Atomic force microscopy and Monte Carlo simulations have shown that at the  $T_m$  there is extensive coexistence of separate domains of each lipid phase.<sup>17–19</sup> These domains have significantly different hydrophobic thicknesses, resulting in incompatibilities in molecular packing and hydrophobic matching, and therefore greater fluctuations and an increased incidence of defect and pore formation at domain interfaces.<sup>17,20</sup> Experimental support for such behavior comes from the observations that lipid bilayers have the greatest permeability to small solutes<sup>20</sup> and exhibit ion conductivity at the phase transition temperature.<sup>24</sup>

A key aspect of OMP assembly is that the  $\beta$ -barrel must insert across the hydrophobic core of the bilayer. Our data demonstrate that OMPs utilize bilayer defects in their folding and insertion process; thus, the fastest folding will occur in the most defect-prone bilayers. In addition to advancing our understanding of the intrinsic OMP  $\beta$ -barrel folding pathway, these observations also have important implications for elucidating the biological process of OMP assembly and the role of folding factors in the cell. In fact, recent evidence has suggested that one function of the BAM complex is to create local defects in the membrane and thus facilitate OMP folding,<sup>7</sup> and our results support this proposal.

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

Detailed description of materials and methods and supporting figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## ■ AUTHOR INFORMATION

### Corresponding Author

\*E-mail: [karen.fleming@jhu.edu](mailto:karen.fleming@jhu.edu). Phone: (410) 516-7256.

### Funding

This work was supported by grants from the National Science Foundation (NSF) (MCB 0919868 and MCB 141210B) and the National Institutes of Health (R01 GM079440 and T32 GM008403). E.J.D. is a recipient of an NSF graduate research fellowship (DGE-0707427).

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

We thank members of the Fleming lab for many helpful discussions.

## ■ REFERENCES

- (1) Tamm, L. K., et al. (2004) *Biochim. Biophys. Acta* 1666, 250–263.
- (2) Ruiz, N., et al. (2006) *Nat. Rev. Microbiol.* 4, 57–66.
- (3) Kolodziejek, A. M., et al. (2010) *Infect. Immun.* 78, 5233–5243.
- (4) Koronakis, V., et al. (2004) *Annu. Rev. Biochem.* 73, 467–489.
- (5) Denoncin, K., et al. (2012) *Proteomics* 12, 1391–1401.
- (6) McMorran, L. M., et al. (2014) *Arch. Biochem. Biophys.* 564, 265–280.
- (7) Noinaj, N., et al. (2013) *Nature* 501, 385–390.
- (8) Ni, D., et al. (2014) *FASEB J.* 28, 2677–2685.
- (9) Surrey, T., and Jähnig, F. (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89, 7457–7461.
- (10) Surrey, T., and Jähnig, F. (1995) *J. Biol. Chem.* 270, 28199–28203.
- (11) Kleinschmidt, J. H., and Tamm, L. K. (2002) *J. Mol. Biol.* 324, 319–330.
- (12) Burgess, N. K., et al. (2008) *J. Biol. Chem.* 283, 26748–26758.
- (13) Gessmann, D., et al. (2014) *Proc. Natl. Acad. Sci. U.S.A.* 111, 5878–5883.
- (14) Kleinschmidt, J. H., and Tamm, L. K. (1996) *Biochemistry* 35, 12993–13000.
- (15) Paula, S., et al. (1996) *Biophys. J.* 70, 339–348.
- (16) Bennett, W. F., et al. (2014) *Biophys. J.* 106, 210–219.
- (17) Heimburg, T. (2007) in *Thermal Biophysics of Membranes*, pp 289–300, Wiley-VCH, Weinheim, Germany.
- (18) Nielsen, L. K., et al. (2000) *Nature* 404, 352.
- (19) Tokumasu, F., et al. (2002) *J. Electron Microsc.* 51, 1–9.
- (20) Mouritsen, O. G., et al. (1995) in *Permeability and Stability of Lipid Bilayers* (Disalvo, E. A., and Simon, S. A., Eds.) pp 137–160, CRC Press, Boca Raton, FL.
- (21) Caffrey, M., and Hogan, J. (1992) *Chem. Phys. Lipids* 61, 1–109.
- (22) Danoff, E. J., and Fleming, K. G. (2011) *Biophys. Chem.* 159, 194–204.
- (23) Lewis, R. N., et al. (1987) *Biochemistry* 26, 6118–6126.
- (24) Antonov, V. F., et al. (1980) *Nature* 283, 585–586.