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Membrane Elastic Fluctuations and the Insertion and Tilt of β-Barrel Proteins

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ABSTRACT Folding of porin-like β-barrel outer membrane proteins can be achieved in the presence of phospholipid vesicles, and takes place concurrently with incorporation into the membrane. The pronounced dependence found for the insertion of the protein OmpA on membrane thickness (Kleinschmidt, J. H., and L. K. Tamm. 2002. *J. Mol. Biol.* 324:319–330) is analyzed in terms of the effects of out-of-plane elastic fluctuations on the area dilation modulus (Evans, E., and W. Rawicz. 1990. *Phys. Rev. Lett.* 64:2094–2097). For unstrained large unilamellar vesicles, the elastic free energy for membrane insertion is predicted to depend on the fourth power of the membrane thickness. The influence of thermally induced bending fluctuations on the effective tilt of the OmpA β-barrel in disaturated phosphatidylcholine membranes of different thicknesses (Ramakrishnan, M., J. Qu, C. L. Pocanschi, J. H. Kleinschmidt, and D. Marsh. 2005. *Biochemistry.* 44:3515–3523) is also considered. A contribution to the orientational order parameter that scales as the inverse second power of the membrane thickness is predicted.

INTRODUCTION

The insertion of proteins into membranes is fundamental not only to membrane biogenesis and traffic, but also to virus and toxin entry into cells. In refolding experiments with the major outer membrane protein from *Escherichia coli* (OmpA), spontaneous insertion of the porin-like β-barrel domain into lipid bilayers has been demonstrated on dilution of the urea-denatured protein into aqueous lipid suspensions (1–3). The original experiments were carried out with small unilamellar phospholipid vesicles that are produced by sonication (i.e., SUVs). These extremely curved vesicles are highly strained, but were found necessary to achieve spontaneous membrane insertion (see also (4,5)). Subsequently, it was shown that the spontaneous insertion of OmpA into unstrained, large unilamellar vesicles (LUVs) depends sensitively on the membrane thickness (6). OmpA was found to fold and insert into LUVs of disaturated phosphatidylcholines with chain lengths of 12 C-atoms or less, but not into LUVs of dimyristoyl or dioleoyl phosphatidylcholine (diC(14:0)PtdCho and diC(18:1) PtdCho, respectively). Correspondingly, it is found that the effective tilt of the OmpA barrel in aligned membranes increases strongly for lipid chain lengths shorter than that of diC(14:0)PtdCho (7).

Here, we explore the possible role of thermally driven membrane bending fluctuations (see Fig. 1) in the protein insertion process and in the time-averaged effective tilting of the protein in the membrane. The effect of the out-of-plane membrane fluctuations is to reduce the elastic modulus, K_A, for the change in membrane area of unstrained membranes, because these area changes can be absorbed by changes in amplitude of the thermal fluctuations (8). This therefore favors the insertion of transmembrane proteins by reducing any elastic penalty involved in membrane compression. Because the bending fluctuations depend on the membrane flexibility, it is expected that these will be enhanced for thinner membranes which are characterized by reduced values of the bending modulus, k_c (9,10). This thus forms a possible basis for the dependence of the protein insertion and effective tilt on lipid chain length. Comparison of these predictions is made with the results of experimental incorporation studies performed to augment those of Kleinschmidt and Tamm (6) and with the infrared dichroism studies of barrel tilt that are reported in Ramakrishnan et al. (7).

RESULTS AND DISCUSSION

Elastic cost of membrane insertion

Because unstressed membranes are tension-free in their natural state (11), lateral pressure or membrane tension contributes to the energetics for insertion of amphiphilic molecules into the membrane only in second order, via elastic compression of the lipids (see, e.g., (12)). If the protein causes a compression of the lipid area by an amount ΔA, on insertion in the bilayer, its chemical potential at mole fraction X_b in the membrane is given by (12):

$$\mu_b = \mu_b^o + k_B T \ln(X_b) + \tilde{K}_A (\Delta A)^2 / A_P, \quad (1)$$

where $\tilde{K}_A$ is the area dilation modulus allowing for thermal fluctuations, A_P is the membrane area per protein molecule, and other symbols have their usual meaning. The chemical potential of the protein in water is given simply by:

$$\mu_w = \mu_w^o + k_B T \ln(X_w). \quad (2)$$

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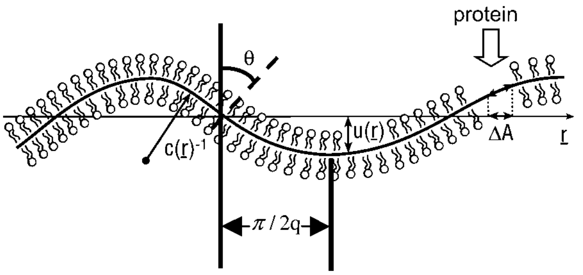

FIGURE 1 Thermal elastic fluctuations of a lipid membrane. The amplitude of the local deviation from the flat membrane is $u(r)$ and the wave vector of one of the independent constituent harmonic modes is q. The local director tilt is θ, which is equal to the angle that the local membrane plane makes with the horizontal.

At equilibrium partitioning, these two chemical potentials are equal. The mole-fraction partition coefficient for protein insertion into the membrane, $K_P = X_b/X_w$, is therefore given by:

$$K_P = K_o \exp\left(\frac{-\tilde{K}_A (\Delta A)^2}{k_B T A_P}\right), \tag{3}$$

where K_o is the notional value of K_P in the absence of compression of the lipids. Insertion of the protein into the membrane is therefore expected to decrease exponentially with increasing values of the renormalized area expansion modulus, $\tilde{K}_A$.

Renormalized membrane expansion modulus

The effect of membrane fluctuations is to renormalize the area expansion modulus, K_A, to an extent that depends sensitively on the bending modulus, k_c (8). In the plane-wave approximation, for a tension-free membrane, the renormalized elastic modulus is (13):

$$\tilde{K}_A = \frac{K_A}{1 + \dfrac{K_A k_B T A}{8\pi^3 k_c^2}} \approx \frac{8\pi^3 k_c^2}{k_B T A}, \tag{4}$$

where A is the total membrane area of the vesicle. The second equality holds for $K_A k_B T A / 8\pi^3 k_c^2 \gg 1$, i.e., when $A \gg 10^4$ nm^2, which is at the limit for LUVs of 100 nm diameter. The curvature modulus scales as $k_c \sim (1/4) K_A d^2$, where d is the thickness of the hydrophobic region of the bilayer (9,10). (The exact numerical prefactor depends on the distribution of lateral pressure across the bilayer (14,15).) Therefore, from Eq. 4, the renormalized expansion modulus scales as the fourth power of the membrane thickness:

$$\tilde{K}_A \approx \frac{\pi^3 K_A^2}{2 k_B T A} d^4 \approx \frac{\pi^3 K_A^2 (\Delta d)^4}{2 k_B T A} (N_c - 1)^4, \tag{5}$$

where Δd is the increment in membrane thickness per lipid CH$_2$ group, and N_C is the number of C-atoms in the lipid chains. This extremely steep dependence on the lipid chain length therefore could possibly account for the high sensitivity of spontaneous insertion of OmpA to membrane thickness. Note that, for realistic membrane tensions, Eqs. 4 and 5 are insensitive to the precise value of the short-wavelength cutoff for the elastic fluctuations (13).

Chain-length dependence for spontaneous membrane insertion of OmpA

Combining Eqs. 3 and 5, the contribution of bending fluctuations to the chain-length dependence of the partition coefficient is given by:

$$K_P \approx K_o \exp\left(-\pi^3 (\Delta d)^4 \left(\frac{K_A}{k_B T}\right)^2 \left(\frac{\Delta A}{a_L}\right)^2 \left(\frac{X_P}{n_L}\right) (N_C - 1)^4\right), \tag{6}$$

where n_L is the number of lipids in a vesicle monolayer, and a_L is the membrane surface area per lipid molecule, i.e., $A = n_L a_L$ and $A_P \approx a_L/(2X_P)$, for $X_P \ll 1$. The partition coefficient therefore depends both on the vesicle size (via n_L or A), and on the final mole fraction of protein in the membrane, X_P. Typical values for the other quantities are: $a_L \approx 0.6$ nm^2, $\Delta d \approx 0.2$ nm/CH$_2$, and $K_A \approx 140$ mN.m^{-1} (16). These values correspond to a curvature modulus of $k_c \sim 2 \times 10^{-19}$ J for diC(14:0)PtdCho, which is in reasonable agreement with experiment (17).

We have performed further refolding experiments for OmpA with 100 nm extruded vesicles of short-chain phosphatidylcholines at different protein/lipid ratios by using the methods of Kleinschmidt and Tamm (6). Results for a lipid/protein ratio of 100:1 mol/mol are shown in Fig. 2. Quantitative insertion of OmpA into diC(10:0)PtdCho, diC(11:0)PtdCho, and diC(12:0)PtdCho vesicles is achieved at this lipid/protein ratio. Similar results were obtained at a lipid/protein ratio of 200:1, except that the kinetics of incorporation are faster in each case. In contrast, no insertion or folding of OmpA into diC(14:0)PtdCho vesicles was observed at the higher lipid/protein ratio of 400:1 mol/mol, which would be expected to favor incorporation.

The maximum value for the condensation, ΔA, of the lipid membrane area is given by the cross-sectional area of OmpA, if tilting of the barrel is ignored. From the crystal structure of OmpA, the diameter of the cylindrical β-barrel is 2.6 nm (18). Using this, together with the above values, it is predicted from Eq. 6 that the OmpA partition coefficient for diC(10:0)PtdCho bilayers (at $X_P = 1/100$) is reduced by a factor of 2×10^{-9} on increasing the lipid chain length to $N_C = 14$. Correspondingly, the prediction for diC(12:0)PtdCho bilayers (at $X_P = 1/100$) is a reduction in partition coefficient by a factor of 3×10^{-6} for a lipid chain length of $N_C = 14$. These estimates ignore any possible change in K_o (or equivalently in the standard chemical potential, μ_b^o), which might arise from hydrophobic matching or other influences on the energetics and stability of the inserted

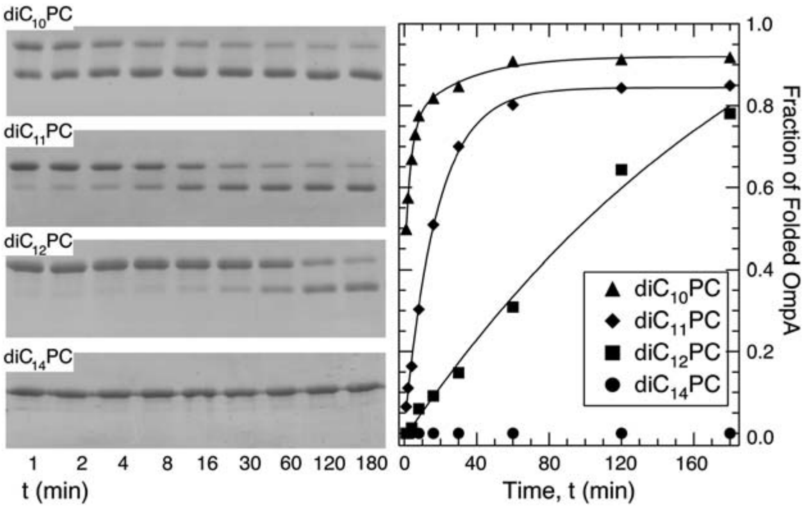

FIGURE 2 OmpA folding into large unilamellar vesicles composed of phosphatidylcholines with different chain lengths: C_{10}, C_{11}, and C_{12}, at a lipid/protein ratio of 100:1 mol/mol; and C_{14} at a lipid/protein ratio of 400:1 mol/mol. (*Left panel*) Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of samples taken at time *t* after dilution of urea-unfolded OmpA (17 μM final concentration) into diC$_{N_C}$PC lipids, at 20°C for $N_C = 10$–12 and 30°C for $N_C = 14$. (*Right panel*) Fractions of folded OmpA obtained by densitometry of the gels in the left panel. For further details of experimental methods, see (6).

protein. Nevertheless, both estimates—which are made for protein/lipid ratios at which insertion is quantitative for the lipid of shorter chain length—are of such a size as to suggest that reduction in the membrane flexibility (and correspondingly in thermal fluctuations) could well be the reason why OmpA is unable to insert spontaneously into LUVs of diC(14:0)PtdCho or lipids of longer chain length, even above the chain-melting transition temperature (see Fig. 2). At the very least, it might be expected that bending fluctuations make a significant contribution to the spontaneous insertion of β-barrel proteins into thin and flexible bilayers, as originally suggested in Kleinschmidt and Tamm (6).

Bending fluctuations and protein tilt

To the extent that the bending fluctuations are not suppressed by the inserted protein, they should give rise to a time-averaged net tilt of the protein, relative to an orienting substrate, for aligned multilamellar samples. The origin of such a tilt is the distribution of local director (i.e., bilayer normal) axes that is depicted schematically in Fig. 1. As such, this type of tilt would not be registered in conventional order parameter measurements on nonaligned samples by magnetic resonance spectroscopy, because these tend to reflect only the time-dependent fluctuations about the local director (cf., e.g., (19)). The lipid chain-length dependence of the OmpA tilt that is determined from polarized infrared spectroscopy of aligned samples mirrors very closely that of the spontaneous protein insertion as described above (7). The tilt of the OmpA β-barrel decreases steeply on increasing the lipid chain length from diC(13:0)PtdCho to diC(14:0)PtdCho and then increases much more gradually with increasing chain length. It is therefore worthwhile to inquire to what extent the lipid chain-length dependence of the effective tilt of membrane-inserted OmpA may be influenced by bending fluctuations in phosphatidylcholine bilayers.

Here, we present a simplified treatment which is intended only to establish how the local director tilt is expected to scale with lipid chain length. From Fig. 1, it is seen that the director tilt angle, θ, is related to the transverse displacement amplitude, $u(r)$, of the fluctuating membrane and the associated wave vector, q, of the fluctuating mode. For small angular amplitudes: $\sin\theta_q \approx 2u_q^{\max} q/\pi$, and the power spectrum of the mean-square displacement amplitudes is given by the equipartition theorem (10,20):

$$\overline{u_q^2} = \frac{k_B T}{A k_c q^4}. \tag{7}$$

An approximate scaling relation for the mean angular fluctuation is therefore:

$$\sin^2\theta_q \approx \frac{8\,k_B T}{\pi^2 A k_c q^2}. \tag{8}$$

Summation over all modes is performed by the usual integration over q-space (21):

$$\sum_q \ldots \rightarrow \frac{A}{(2\pi)^2} \int_{q_{\min}}^{q_{\max}} \ldots \pi d(q^2), \tag{9}$$

where the cutoff wave vectors are given by $q_{\max} \approx \pi/\sqrt{a_L}$ and $q_{\min} \approx \pi/\sqrt{A}$. The approximate result for the order parameter associated with the director fluctuations is then:

$$\langle P_2(\cos\theta)\rangle = \frac{1}{2}(3\langle\cos^2\theta\rangle - 1) \approx 1 - \frac{3\,k_B T}{\pi^3 k_c}\ln\frac{A}{a_L}. \tag{10}$$

This analysis therefore predicts contributions to the orientational order parameter that scale as $1/k_c$, i.e., as the inverse second power of the membrane thickness. In the case of multilayer stacks, A is no longer determined by the vesicle size. The cutoff wave vector is then limited by the transverse excursions of the undulations: $q_{\min} = \pi/\xi_{//}$. The correlation length is then given by $\xi_{//} \sim \sqrt{(k_c/k_B T)}.d$, where d is the interlayer spacing (22–24). Note that the precise choice of values for the cutoff wave vectors does not change the functional dependence on lipid chain length.

Fig. 3 shows the chain-length dependence of the order parameters of the β-barrel domain of OmpA in aligned membranes of diC(N_C:0)PtdCho lipids. The steep increase in order parameter between diC(13:0)PtdCho and diC(14:0)PtdCho is clearly evident. The solid line in the figure represents a nonlinear least-squares fit of a function: $P_2 \sim 1 - B/(N_C - 1)^2$, which has the chain-length dependence that is predicted by Eq. 10 for the director fluctuations. Clearly there are contributions other than fluctuations of the lipid director to the tilt of OmpA in phosphatidylcholine membranes; for instance from hydrophobic mismatch to which originally we attributed the chain-length dependence in disaturated phosphatidylcholines (7). Nonetheless, the phenomenological fit given in Fig. 3 suggests that bending fluctuations of the membrane could make significant contributions to the steep increase in tilt of the protein that is found for lipid chain lengths lying in the region of those expected to give hydrophobic matching with OmpA (cf. (7)).

For comparison, simple geometric hydrophobic matching predicts that the tilt of OmpA relative to the bilayer normal is given by:

$$\cos\theta = \frac{\Delta d}{d_p}(N_C - 1), \qquad (11)$$

where d_p is the hydrophobic thickness of OmpA. For membranes of thickness less than d_p, the order parameter would therefore increase with the square of the lipid chain length. A nonlinear least-squares fit of a function with the form $P_2 \sim B'(N_C - 1)^2 - 1/2$, is included as an illustration in Fig. 3. This yields $P_2 = 1$ for $N_C \cong 18$, which rather exceeds the hydrophobic thickness of OmpA. Hydrophobic matching most probably makes significant contributions to the tilt of β-barrel proteins, but is unlikely to make the dominant contributions to the chain-length dependence of the tilt in disaturated phosphatidylcholines.

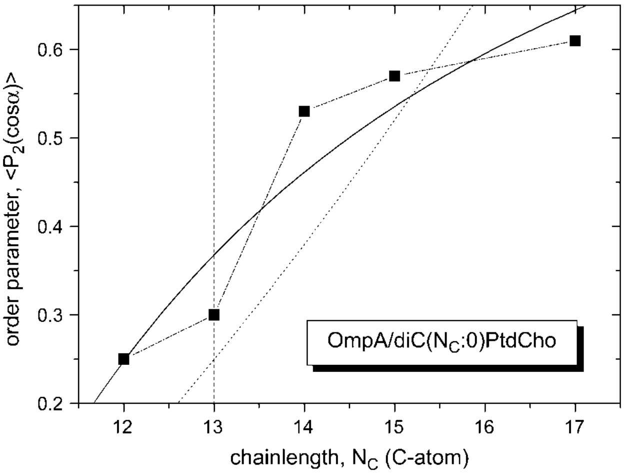

FIGURE 3 Dependence of the order parameters $\langle P_2(\cos\alpha)\rangle$ of the β-barrel domain of OmpA on the chainlength, N_C, of the disaturated phosphatidylcholine fluid-phase membrane in which OmpA is incorporated (data from (7)). The solid line represents a nonlinear least-squares fit ($\chi^2 = 0.003$) of a chain-length dependence with the form given by Eq. 10. The vertical dashed line corresponds to the lipid chain length that gives approximate hydrophobic matching with OmpA. The continuous dotted line is a nonlinear least-squares fit ($\chi^2 = 0.023$) for simple geometric hydrophobic matching (Eq. 11), which yields $\langle P_2(\cos\alpha)\rangle = 1$ for $N_c = 18$.

Effects of intrinsic lipid curvature

In this final section, we consider the possible influence of intrinsic lipid curvature, because this depends on lipid chain length and also manifests its energetic effects via the bending modulus, k_c. At those parts of the intramembranous surface where the central region between the two aromatic belts is not filled up by bulky hydrophobic side chains, OmpA presents an approximately hourglass-shaped transmembrane profile to the lipid chains (see Fig. 4 and (25)). Membrane insertion of OmpA, if affected by lipid curvature, would therefore be favored by lipids with negative intrinsic curvature, which tend to form inverted lyotropic phases. Such a mechanism could not explain the chain-length dependence for spontaneous insertion of OmpA into LUVs of disaturated phosphatidylcholines because decreasing chain length reduces negative intrinsic curvature (26) or favors positive intrinsic curvature (27). Note also that OmpA is unable to insert spontaneously into LUVs of diC(18:1)PtdCho, which has a membrane thickness similar to that of diC(14:0)PtdCho (28), but a larger lipid cross-sectional area that is expected to produce a more pronounced negative intrinsic curvature (29).

Although unable to explain the direction of the chain-length dependence for OmpA insertion, it is instructive to consider the energetic consequences that intrinsic lipid curvature might have on protein insertion. The contribution of local elastic curvature free energy to the chemical potential of the protein in the membrane (i.e., to Eq. 1) is given by (see, e.g., (10)):

$$\Delta\mu_b^{\mathrm{curv}} = \frac{1}{2} n_b a_L k_c (c - c_o)^2, \qquad (12)$$

where n_b is the number of lipids at the intramembranous perimeter of the protein, c is the effective local membrane curvature of these lipids, and c_o is their spontaneous or intrinsic curvature. The latter can be expressed in terms of the dimensions of the (fluid) lipid molecules by (29):

$$c_o = \frac{1}{R_o} = \frac{2}{l}\left(1 - \frac{V}{Al}\right), \qquad (13)$$

where V is the total volume and l the total length of the lipid molecule, and A is the molecular area at the lipid-water interface. The packing parameter (V/Al) in Eq. 13 is close to unity for phosphatidylcholines of moderate chain length

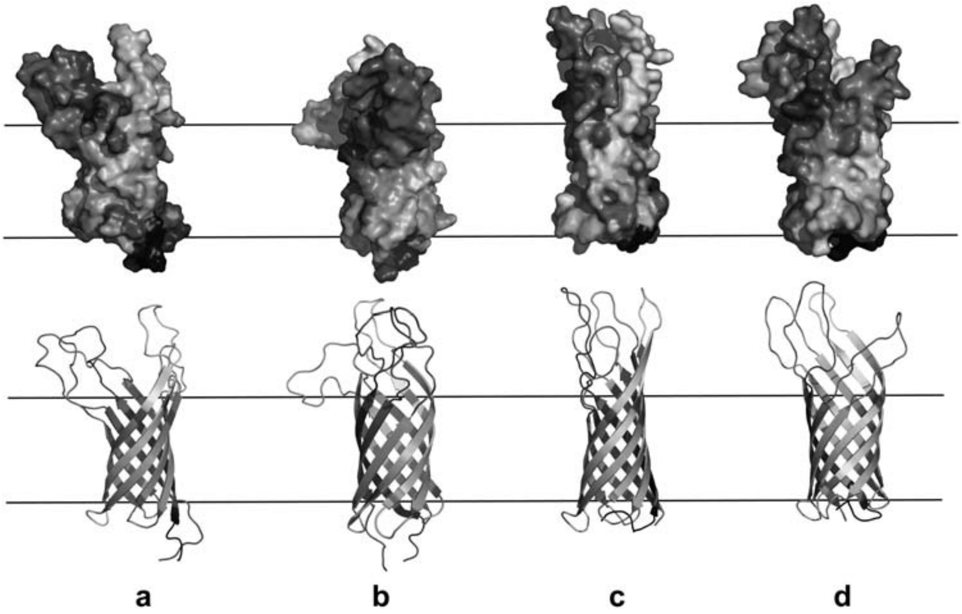

FIGURE 4 Van der Waals surfaces of OmpA: (*a* and *b*) NMR solution structure (Protein Data Bank code 1G90 (32)); (*c* and *d*) x-ray crystal structure (Protein Data Bank code 1BXW (18)). Surfaces *a* and *b*, and *c* and *d*, represent orthogonal views from within the membrane plane. Horizontal lines indicate the approximate location of the polar-apolar interface.

(29). In terms of the hydrophobic membrane thickness introduced previously, the length of the lipid molecule is given by:

$$l = l_{\mathrm{HG}} + d = l_{\mathrm{HG}} + (N_{\mathrm{c}} - 1)\Delta d, \tag{14}$$

where l_{HG} is the thickness of the lipid headgroup region. The sign convention in Eq. 13 is that lipids favoring inverted lyotropic phases have negative curvature.

As regards protein insertion, the elastic curvature will be energetically unfavorable if the intrinsic lipid curvature, c_{o}, does not match the value (c_{p}) which is required for the lipid to fit best at the protein surface. The maximum elastic stabilization, relative to a lipid with $c_{\mathrm{o}} = 0$, is therefore: $\Delta\Delta\mu_{\mathrm{b}}^{\mathrm{curv}} = -(1/2)n_{\mathrm{b}}a_{\mathrm{L}}k_{\mathrm{c}}c_{\mathrm{o}}^2$, for $c_{\mathrm{o}} = c_{\mathrm{p}}$. Thus, the chain-length dependence of the intrinsic curvature (Eq. 13) tends to compensate that of the bending modulus, in the curvature energy. The end result is that the stabilization of the curvature free energy is given by:

$$\Delta\Delta\mu_{\mathrm{b}}^{\mathrm{curv}} = -n_{\mathrm{b}}a_{\mathrm{L}}K_{\mathrm{A}}\left(\frac{1 - V/Al}{1 + l_{\mathrm{HG}}/d}\right)^2. \tag{15}$$

Because $V/Al \approx 1$ for phosphatidylcholines, the stabilization is intrinsically small for this lipid species; and the chain-length dependence of the denominator in Eq. 15 is not large for $d > l_{\mathrm{HG}}$. If the radius of curvature is referred to the polar-apolar interface, which is close to the neutral or pivotal plane, rather than to the lipid-water interface, then this term in l_{HG} even drops out entirely (cf. (30)). Thus, the chain-length dependence of the contribution from intrinsic curvature to the chemical potential of the inserted protein is expected to be much smaller than that from bending fluctuations.

CONCLUSIONS

The analysis given here suggests that membrane flexibility, in the form of out-of-plane bending fluctuations, may well play a very significant role in the spontaneous insertion and folding of β-barrel proteins into lipid membranes. This arises from the very strong dependence on membrane thickness that is predicted for the elastic free energy cost of lateral membrane compression accompanying protein insertion. In comparison, transverse elastic membrane distortions that arise from hydrophobic mismatch are expected to have a free energy that depends only quadratically on the membrane thickness (see, e.g., (31)). For somewhat similar reasons, thermally excited lipid director fluctuations additionally may make significant contributions to the time-average effective tilt of β-barrels in membranes. In contrast, a simple geometric model of tilt induced to achieve hydrophobic matching would predict a direct quadratic dependence of order parameter on membrane thickness, and that an order parameter of unity (i.e., zero tilt) would be reached already for the lipid chain length that corresponds to hydrophobic matching. These latter predictions deviate considerably from the data presented in Fig. 3.

Finally, it might be surmised that the kinetics of protein insertion and folding may also depend on the intensity of the out-of-plane membrane fluctuations. The rate constants for insertion and folding of OmpA increase very appreciably on decreasing the lipid chain length (see Fig. 2, and (6)), and Eq. 7 predicts an inverse quadratic dependence of the mean-square fluctuation amplitudes on membrane thickness.

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