Curvature Elasticity and Refolding of OmpA in Large Unilamellar Vesicles

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ABSTRACT The stability of OmpA in large unilamellar vesicles of dilauroyl phosphatidylcholine was studied using different concentrations of urea. The effective energy of unfolding, as determined from refolding experiments, is greater than that for small sonicated unilamellar vesicles by an amount that is compatible with estimates of the elastic energy of highly curved vesicles. The on-rate for refolding and insertion is slower for large unilamellar vesicles than for small unilamellar vesicles, which indicates a contribution of vesicle strain also to the free energy of the transition state.

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Certain β-barrel outer-membrane proteins from Gram-negative bacteria that are unfolded in urea are able, on dilution, to insert spontaneously into membranes of a variety of lipids, provided that they are in the form of small unilamellar vesicles (SUVs), which are produced by limit sonication (1–3). However, the major outer membrane protein from *Escherichia coli* (OmpA), for instance, is unable to insert spontaneously into large unilamellar vesicles (LUVs), unless they are composed of short-chain phospholipids ($n \leq 12$), which form highly flexible membranes (2,4).

In an elegant series of experiments, Hong and Tamm (5) established conditions for the reversible folding of OmpA in SUVs, and used this to investigate the thermodynamic stability of OmpA in a systematic range of phospholipids. Because of the dramatic differences in potential for spontaneous protein insertion between LUVs and SUVs, it is of interest to attempt studies of the stability of OmpA in LUVs. This is done here, together with consideration of the contribution of vesicle curvature elasticity to protein stability, which can at least partially explain the much higher stability of OmpA in LUVs than in SUVs.

Fig. 1 shows refolding curves for insertion of OmpA into extruded LUVs, from different concentrations of aqueous urea (solid symbols). Qualitatively similar results are obtained from LUVs of dilauroyl phosphatidylcholine ($diC_{12}PC$) and of a 1:1 mol/mol mixture of $diC_{12}PC$ with dilauroyl phosphatidylglycerol ($diC_{12}PG$). The effective free energies of folding, obtained according to Fig. 1, are 22 ± 2 and 16 ± 2 kJ.mol^{-1} ($m' \approx 3.4$ and 3.3 M^{-1}) for refolding into $diC_{12}PC$ and into $diC_{12}PC$:$diC_{12}PG$, respectively, after a 1-day incubation. These values decrease to effective unfolding energies of $\Delta G_u = 15 \pm 5$ and 13 ± 5 kJ.mol^{-1} ($m' \approx 2.3$ and 2.5 M^{-1}), respectively, after an incubation of 12 days. On the other hand, incubation in up to 9.6 M urea for 12 days at 40°C does not induce any detectable unfolding of OmpA that is already inserted in LUVs of either lipid composition (open symbols in Fig. 1).

These results for LUVs differ considerably from those obtained with sonicated SUVs by Hong and Tamm (5). In the latter case, reversible folding and unfolding of OmpA was demonstrated on overnight incubation in urea at pH 10.0 and a temperature of 37.5°C. Inclusion of at least 7.5 mol % of PG in the SUVs was required to obtain these results. Also, the free energy and cooperativity of folding were found to be considerably lower than the effective values obtained here in refolding experiments with LUVs. The reversible free energy of unfolding of OmpA in SUVs at 37.5°C, extrapolated to 92.5% $diC_{12}PC$ plus 7.5% 1-palmitoyl-2-oleoyl PG, amounts to only $\Delta G_u^o \approx 6$ kJ.mol^{-1} ($m' = 0.2$ M^{-1}) (5). Vesicle curvature therefore has a very significant effect on the stability of OmpA in SUVs of $diC_{12}PC$.

The free energy of bending that is associated with each monolayer of a curved vesicle is given by (7)

$$\Delta G_c^{(m)}(c_1, c_2) = \frac{1}{2} k_c A_m (c_1 + c_2 - c_o)^2 + \bar{k}_c A_m c_1 c_2, \quad (1)$$

where c_1 and c_2 are the principal curvatures, k_c and $\bar{k}_c$ are the elastic moduli of mean and Gaussian curvature of the monolayer, respectively, c_o is the spontaneous curvature of the constituent lipid monolayers, and A_m is the area of the monolayer surface at the neutral plane. For a spherical vesicle, the areas at the neutral surfaces of the outer and inner monolayers are given, respectively, by:

$$A_{out/in} = A(1 \pm 2\delta c + \delta^2 c^2), \quad (2)$$

where A and c ($= 1/R_{ves}$) are the area and curvature, respectively, at the bilayer midplane, and $\pm\delta$ are the distances of the neutral surfaces from the midplane (see Fig. 2). Correspondingly, the curvatures at the neutral planes of the outer and inner monolayers of a spherical vesicle are given, respectively, by:

$$c_{out/in,1} = c_{out/in,2} = \pm(R_{ves} \pm \delta)^{-1} = \pm c/(1 \pm \delta c), \quad (3)$$

where an outward curvature is defined as positive, and an inward curvature (as in lipid H_{II} phases) is negative.

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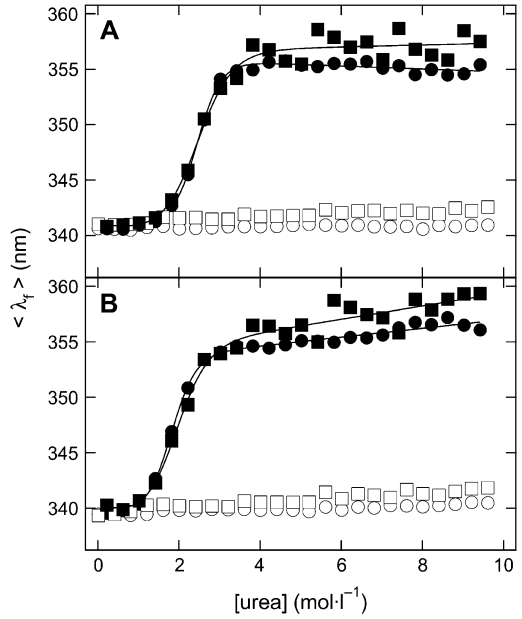

FIGURE 1 Refolding (*solid symbols*) and denaturation (*open symbols*) curves of OmpA with LUVs (lipid/protein 800 mol/mol) of (*A*) $diC_{12}PC$ and (*B*) $diC_{12}PC/diC_{12}PG$ 1:1 mol/mol. Samples were incubated at 40°C in 10 mM borax, 2 mM EDTA, pH 10.0 for 1 day (*circles*), or 12 days (*squares*). Average tryptophan fluorescence emission wavelength, $\langle\lambda\rangle = (\langle\lambda\rangle_F + \langle\lambda\rangle_U Q_R^{-1}\exp(m'[\text{urea}] - \Delta G_u^o/RT))/(1 + Q_R^{-1}\exp(m'[\text{urea}] - \Delta G_u^o/RT))$, is plotted against denaturant concentration, [urea]. For a two-state process, ΔG_U is the free energy of unfolding in water alone, where $\langle\lambda\rangle_F$ and $\langle\lambda\rangle_U$ are average emission wavelengths, and Q_R is the ratio of the fluorescence intensities, for the folded and unfolded protein, respectively (5,6).

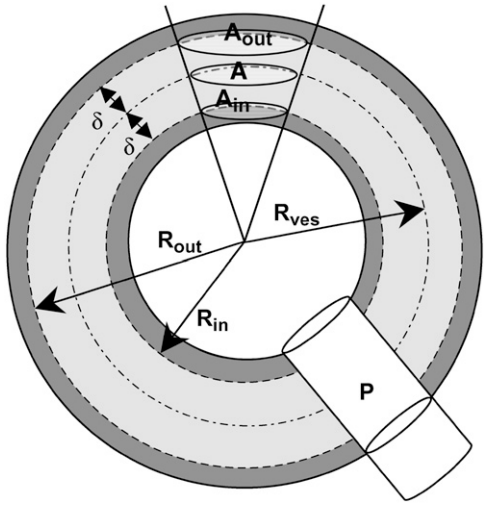

FIGURE 2 Geometry of outer (*out*) and inner (*in*) monolayers in a spherical vesicle. Curvatures of the two neutral surfaces are given by $c_{out} = 1/(R_{ves} + \delta)$ and $c_{in} = -1/(R_{ves} - \delta)$. Areas at the two neutral surfaces are $A_{out} = A(1 + \delta c)^2$ and $A_{in} = A(1 - \delta c)^2$, where $c = 1/R_{ves}$ is the curvature of the vesicle. The asymmetric environment in which a transmembrane protein, *P*, finds itself in a SUV is indicated schematically by the different degree of projection from the vesicle surface in the outer and inner monolayers.

Summing free energy contributions from the outer and inner monolayers of the bilayer membrane (taking due account of both sign and magnitude of the different curvatures, and of the different numbers of lipids in the two monolayers) yields the following result for the bending free energy of a spherical vesicle:

$$\Delta G_c^{(ves)}(c) = k_c A c^2(4 + 2\bar{k}_c/k_c - 4\delta c_o + \delta^2 c_o^2). \qquad (4)$$

This value is expressed relative to a planar bilayer of the same area, for which $c = 0$ and the elastic curvature energy is simply $\Delta G_c^{(b)}(0) = k_c A c_o^2$. For SUVs with diameters of 30 nm, this elastic energy corresponds to ~950 kJ/mol, assuming values typical for dioleoyl phosphatidylcholine ($diC_{18:1}PC$) of $k_c \approx 10\,k_BT$, $\bar{k}_c/k_c \approx -0.8$, $c_o \approx -0.1\ nm^{-1}$, and $\delta \approx 1.5$ nm for a monolayer (8–11). This is a very conservative estimate because sonicated vesicles are highly strained and the effective bending rigidity, under these conditions, is possibly much higher.

In a fluid bilayer of $diC_{18:1}PC$, the area per lipid molecule is $A_L \approx 0.72\ nm^2$ (12). The curvature free energy of $diC_{18:1}PC$ SUVs thus amounts to minimally ~120 $J.mol^{-1}$ per lipid molecule. The cross-sectional area of OmpA corresponds to ~15 lipid molecules (13), and the number of first-shell lipids that can be accommodated around the intramembranous perimeter is $n_b \approx 20$ (14). Perturbations of lipid curvature by incorporation and folding of OmpA therefore can influence the protein stability in SUVs appreciably. Alleviation of curvature stress by protein incorporation in SUVs of $diC_{18:1}PC$ would hence tend to stabilize the inserted protein. This is in accordance with the observation that OmpA inserts spontaneously into SUVs of $diC_{18:1}$ (or dimyristoyl) PC, but not into LUVs composed of either of these lipids (2,4).

As seen from Eq. 4, the elastic free energy of SUVs depends directly on the spontaneous curvature, c_o, of the constituent lipids. For lipids with $\delta c_o > 2 - \sqrt{-2\bar{k}_c/k_c}$ (~+0.4 nm^{-1}), the sign of the overall bending energy changes and becomes negative. The reason for this is that the outer monolayer of an SUV, which contains 50% more lipids than the inner monolayer, has a positive curvature. There are no experimental measurements of the spontaneous curvature for $diC_{12}PC$. However, considerations of lipid molecular shape suggest that the spontaneous curvature for $diC_{12}PC$ is positive and greater in absolute magnitude than that for $diC_{18:1}PC$ (9,15). It is therefore perfectly conceivable that incorporation of OmpA in SUVs of $diC_{12}PC$ could be unfavorable as regards bending free energy of the vesicle and hence destabilize the protein relative to the state in LUVs.

There is another aspect of the transbilayer asymmetry in SUVs that also could affect the stability of inserted proteins. An approximately cylindrical β-barrel protein is not readily compatible with the transmembrane lipid packing in SUVs (see Fig. 2), particularly at the inner monolayer, which is less extensive ($A_{in} < A_{out}$) and more highly curved ($|c_{in}| > |c_{out}|$) than is the outer monolayer. Almost certainly, protein

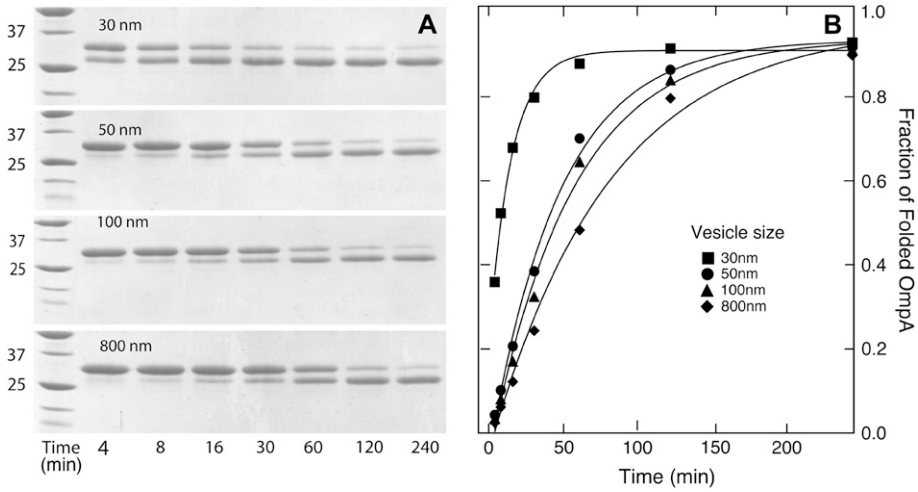

FIGURE 3 Folding kinetics of OmpA into diC_{12}PC vesicles of different diameters at 20°C. (*A*) SDS-PAGE of OmpA (17 μM) at different times of incubation after dilution from urea into 7 mM lipid with vesicle diameters indicated. Folding is accompanied by a downward gel shift to lower effective molecular mass. (*b*) Fraction of folded OmpA with time determined from gel densitometry. For further details of methods, see Kleinschmidt and Tamm (2).

incorporation will require transfer of some lipid molecules between the two monolayers. The change in curvature free energy on transfer of a lipid molecule from the inner to the outer monolayer is $-4\ k_c A_L c c_o$ per lipid, according to Eqs. 1 and 3. This amounts to ~ -5.0 kJ.mol^{-1}.nm $\times\ c_o$ for a 30-nm vesicle and is determined directly by the magnitude and sign of the intrinsic curvature, c_o, of the lipid molecule. This contribution from vesicle rearrangement may therefore have either a stabilizing or a destabilizing effect depending on the particular lipid and on the direction of lipid transfer.

It is thus quite possible that the above effects of curvature elasticity could together account for the apparent destabilization (i.e., reduction in unfolding energy) of OmpA in SUVs, relative to LUVs, which is $\Delta\Delta G_u \approx -(7\text{–}9 \pm 5)$ kJ.mol^{-1}. In this connection, it should be taken into account that the effective bending rigidity for highly curved, sonicated SUVs may be considerably larger than assumed here (see above) and that equilibrium refolding might not have been achieved in LUVs, even after such a long incubation time.

The unfolding energy ΔG_u contributes also to the activation free energy for unfolding: $\Delta G_u^{\ddagger} = \Delta G_f^{\ddagger} + \Delta G_u$, where $\Delta G_f^{\ddagger}$ is the activation free energy for folding. A change of the size measured here, i.e., $\Delta\Delta G_u = 7\text{–}9$ kJ.mol^{-1} relative to SUVs, would decrease the unfolding rate by a factor of $\leq$30-fold. This alone would be insufficient to account for the very slow (i.e., undetectable) unfolding rates in LUVs, relative to SUVs. Most probably also the free energy of the transition state $\Delta G_f^{\ddagger}$ is reduced in SUVs, relative to LUVs, which should then be reflected in the on-rates. Fig. 3 shows results on folding kinetics for insertion of OmpA into diC_{12}PC vesicles of different diameters. The forward rate constant is 0.067 min^{-1} for 30-nm sonicated SUVs, and has a mean value of 0.013 ± 0.004 min^{-1} for extruded LUVs of diameter 100 nm and greater. Taken together with the experimental difference in unfolding energy, this factor of 5$\times$ in on-rate would predict a difference in off-rates (i.e., in rates of unfolding) of maximally 150-fold. This could be sufficient to explain the difference between overnight incubation of SUVs and a 12-day incubation of LUVs, i.e., the apparent irreversibility of folding in LUVs may simply be a kinetic limitation.

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