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Monitoring Lipid Membrane Translocation of Sodium Dodecyl Sulfate by Isothermal Titration Calorimetry

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Abstract: We establish high-sensitivity isothermal titration calorimetry (ITC) as a fast, reliable, and versatile tool for assessing membrane translocation of charged compounds. A combination of ITC uptake and release titrations can discriminate between the two extreme cases of half-sided binding and complete transbilayer equilibration on the experimental time scale. To this end, we derive a general fit function for both assays that allows for incorporation of different membrane partitioning models. Electrostatic effects are taken into account with the aid of Gouy−Chapman theory, thus rendering uptake and release experiments amenable to the investigation of charged solutes. This is exemplified for the flip−flop of the anionic detergent sodium dodecyl sulfate (SDS) across the membranes of 100-nm-diameter unilamellar vesicles composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) in aqueous solution (10 mM phosphate buffer, 154 mM NaCl, pH 7.4). If repulsive electrostatic forces are accounted for adequately, SDS binding to POPC membranes can be evaluated on the basis of ideal mixing in all phases. At 25 °C, the intrinsic partition coefficient between the interfacial aqueous phase and the membrane amounts to 3.5×10^6; however, detergent flip−flop is negligibly slow under these conditions. Raising the temperature to 65 °C lowers the intrinsic partition coefficient to 1.4×10^6 but enables rapid transbilayer distribution of the detergent and, therefore, binding to or desorption from both membrane leaflets. Thus, combining a surface partition equilibrium with simple electrostatic theory appears highly useful in monitoring transmembrane movement of ionic compounds by ITC, thereby eliminating the need for specific reporter groups.

Introduction

The ability of a molecule to traverse a lipid bilayer is of paramount importance not only to the determination of membrane partition equilibria[1] but also to the extracellular administration of intracellularly active agents in cell biology and drug development. Even though a broad range of techniques are available for assessing transbilayer distribution and membrane permeability, most of them depend on specific traceable properties of the compound at hand,[2,3] require introduction of an extrinsic reporter,[4,5] or are expensive and time-consuming.

High-sensitivity isothermal titration calorimetry[6] (ITC) offers the great advantage of relying on a nearly ubiquitous signal, that is, the enthalpy change accompanying a reaction. In particular, a classical "uptake" protocol[7−13] has proven useful in the rapid and comprehensive thermodynamic characterization of membrane partitioning without, however, providing information on bilayer translocation. To this end, an ITC "release" assay[8,14−16] has been put forward which senses the heat of desorption arising from the dilution of lipid vesicles preloaded on both leaflets with the compound of interest. Taken together, uptake and release measurements allow for a reliable quantification of flip−flop across the bilayer; unfortunately, the applicability of this method has remained restricted to nonionic molecules because electrostatic effects at the membrane surface elude the rationale underlying the original release protocol.

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Although ions usually cannot quickly overcome a lipid barrier by diffusion across the hydrophobic membrane core, flip−flop of charged molecules is attracting considerable attention as many biologically and pharmacologically relevant compounds have pK_a values in the vicinity of physiological pH ranges, thus conferring membrane permeability upon (de)protonation. Furthermore, the recent years have witnessed an increasing number of biomolecules that pass through a membrane in nondiffusive ways, such as venomous,[17] antimicrobial,[18−20] or so-called cell-penetrating peptides.[21]

Here, we demonstrate that, based on a simple surface partition equilibrium and Gouy−Chapman theory, ITC constitutes an excellent tool for monitoring transbilayer movement of charged compounds, such as ionic detergents. Sodium dodecyl sulfate (SDS) is a negatively charged surfactant frequently used for membrane solubilization, protein purification and reconstitution, and many other applications. SDS micelle formation,[22−24] partitioning into monolayers[25] and bilayers,[22,24] and interactions with membrane proteins[2] have been characterized in detail. Experiments employing radiolabeled detergent[2] or a fluorescent membrane probe[4] have shown that SDS permeates a lipid bilayer only very slowly at room temperature, whereas solubilization studies[22,24] have implied rapid flip−flop at and above 50 °C. Hence, SDS lends itself as an ideal model system for our purpose because variation of a single parameter, namely, temperature, affords conditions for either half-sided binding or fast transbilayer equilibration.

We therefore performed ITC uptake and release titrations over a broad temperature range to assess SDS flip−flop across the membranes of large unilamellar vesicles (LUVs) made up of the zwitterionic phospholipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) in aqueous solution (10 mM phosphate buffer, 154 mM NaCl, pH 7.4). In full agreement with previous findings, the results demonstrate rapid transbilayer movement at 65 °C, but not at 25 °C, and yield a consistent picture of SDS partitioning into and translocation across POPC vesicles, thus validating ITC as a powerful and label-free method for monitoring membrane flip−flop of charged compounds.

Theory

General Fit Function. Different ways of evaluating ITC membrane partitioning experiments have been developed. A method based on cumulative heats[8,12,13] can easily accommodate electrostatic effects but is less precise than other approaches and is restricted to the uptake mode. Using partial derivatives of the concentration of bound solute[7,8,11] is more accurate and widely applicable but cannot account for electrostatics because there exists no explicit function reflecting the dependence of the apparent partition coefficient on the concentrations of lipid and charged solute (cf. eqs 11 and 13 in ref 14). A formalism employing finite concentration differences[9,10] combines the advantages of the aforementioned methods in that it is precise, generic, and adaptable to ions. However, all approaches thus far presented for the release protocol[8,14−16] depend on partial derivatives and are therefore limited to nonionic compounds. The universal fit function derived in the following relies on finite concentration differences and can thus be used for the assessment of membrane translocation of both neutral and charged molecules.

The normalized heats produced or consumed in an uptake or a release experiment, Q_L and Q_{L+D}, respectively, are given by

$$Q_{L;L+D} = \Delta n_D{}^b \frac{\Delta H_D{}^{b/aq}}{\Delta n_L} + Q_{L;L+D,dil} = V \Delta c_D{}^b \frac{\Delta H_D{}^{b/aq}}{\Delta n_L} + Q_{L;L+D,dil} \quad (1)$$

where $\Delta H_D{}^{b/aq}$ stands for the molar transfer enthalpy of the detergent (D; here, SDS) from the aqueous (aq) into the bilayer (b) phase, V for the volume of the calorimeter cell, and $Q_{L,dil}$ ($Q_{L+D,dil}$) for the heat of dilution in an uptake (release) experiment normalized with respect to the molar amount of lipid (L; here, POPC) injected, Δn_L. $\Delta n_D{}^b$ and $\Delta c_D{}^b$ are the respective changes in the molar amount and the concentration of membrane-bound detergent during reequilibration after injection. Let $\hat{c}_D{}^b$ and $c_D{}^b$ be the equilibrium concentrations of bound detergent before and after an injection of volume ΔV, respectively, and $c_D{}^{b,s}$ that in the syringe (s; $c_D{}^{b,s} = 0$ for uptake experiments). $\Delta c_D{}^b$ can then be expressed as a volume-weighted difference,

$$\Delta c_D{}^b = c_D{}^b - \left(1 - \frac{\Delta V}{V}\right)\hat{c}_D{}^b - \frac{\Delta V}{V} c_D{}^{b,s} \quad (2)$$

This equation is based on the assumption that the injection of ΔV from the syringe into the calorimeter cell and the concomitant expulsion of ΔV from the cell into the calorimetrically inert access tube are finished before the injectant mixes with the cell content. This is in conflict with the equations used by the ITC manufacturer (MicroCal, Northampton, U.S.A.) to correct the total detergent and lipid concentrations in the cell, c_D and c_L, respectively, for dilution effects. However, the resulting errors are negligible as long as $\Delta V/V \ll 1$ and, for the examples presented here, amount to <1%. A general fit function for both uptake and release titrations is hence obtained upon inserting eq 2 into eq 1 as

$$Q_{L;L+D} = V\left(c_D{}^b - \left(1 - \frac{\Delta V}{V}\right)\hat{c}_D{}^b - \frac{\Delta V}{V} c_D{}^{b,s}\right)\frac{\Delta H_D{}^{b/aq}}{\Delta n_L} + Q_{L;L+D,dil} \quad (3)$$

The explicit expressions for the concentrations of bound detergent, $c_D{}^b$, $\hat{c}_D{}^b$, and $c_D{}^{b,s}$, depend on the thermodynamic model used to describe membrane partitioning, the most obvious of which is delineated in the following. Another common and straightforward approach is described in detail in the Supporting

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Information. For a thorough discussion of different membrane partitioning models, consult refs 8 and 26.

Ideal Mixing. Physically, the simplest membrane partitioning model[7,9−11,14] assumes ideal mixing in both the aqueous solution and the membrane phase and is characterized by a mole fraction partition coefficient defined as

$$K_D^{b/aq} \equiv \frac{X_D^{\ b}}{X_D^{\ aq}} = \frac{c_D^{\ b}(c_W + c_D^{\ aq})}{c_D^{\ aq}(c_L + c_D^{\ b})} \quad (4)$$

Here, $X_D^b \equiv c_D^b/(c_L + c_D^b)$ and $X_D^{aq} \equiv c_D^{aq}/(c_W + c_D^{aq})$ stand for the mole fractions of detergent in the membrane and in the bulk aqueous phase, respectively, and c_D^b, c_D^{aq}, c_L, and c_W are the molar concentrations of membrane-bound and free detergent, lipid, and water (W), respectively. Neglecting c_D^{aq} as compared with $c_W = 55.5$ M in the numerator and using the total detergent concentration, c_D, to substitute $c_D^{aq} = c_D - c_D^b$ in the denominator, one obtains

$$K_D^{b/aq} = \frac{c_D^{\ b} c_W}{(c_D - c_D^{\ b})(c_L + c_D^{\ b})} \quad (5)$$

which yields the concentration of membrane-bound detergent as

$$c_D^{\ b} = \frac{1}{2K_D^{\ b/aq}}\Big(K_D^{\ b/aq}(c_D - c_L) - c_W + \sqrt{K_D^{\ b/aq^2}(c_D + c_L)^2 - 2K_D^{\ b/aq}(c_D - c_L)c_W + c_W^{\ 2}}\Big) \quad (6)$$

Corresponding equations hold for $\hat{c}_D^b$ and $c_D^{b,s}$. For a charged detergent, substituting c_D^{aq} by $c_D - c_D^b$ is a very good approximation rather than an equality because it does not consider the depletion (or, in the case of oppositely charged lipid headgroups, enrichment) of detergent near the membrane surface.

Transbilayer Distribution. The equations derived above apply to conditions where the detergent can equilibrate across the membrane within the time frame of a single ITC injection, that is on the order of minutes to hours. If this is not the case, the concentration of lipid available for binding in both uptake and release experiments has to be corrected to

$$c_L \rightarrow \gamma c_L \quad (7)$$

where γ denotes the lipid accessibility factor.[8,13−16] The detergent concentration remains unaffected in the uptake protocol; however, it also needs correction in the release mode because only part of the detergent is free to distribute between the membrane and the external aqueous phase. Using the total detergent concentration, c_D^s, and the concentration of membrane-bound detergent, $c_D^{b,s}$, in the syringe, the correction factor may be written as the sum of the fractions of accessible membrane-bound detergent, $\gamma c_D^{b,s}/c_D^s$, and of detergent in the aqueous phase, $1 - c_D^{b,s}/c_D^s$, such that

$$c_D \rightarrow \left(\frac{c_D^{\ b,s}}{c_D^{\ s}}(\gamma - 1) + 1\right)c_D \quad (8)$$

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$c_D^{b,s}$ is obtained from c_D^s with the aid of eq 6 without any corrections because all of the lipid is accessible to all of the detergent during vesicle formation. However, $c_D^{b,s}$ has to be corrected to

$$c_D^{\ b,s} \rightarrow \gamma c_D^{\ b,s} \quad (9)$$

before substitution into eq 3. Equation 8 neglects the detergent that is entrapped in the aqueous lumen of the vesicles. Geometrical considerations yield the ratio of the intravesicular volume, V_{ves}, to the total volume, V_{tot}, as $V_{ves}/V_{tot} \approx N_A c_L A_L r/6$, where N_A is Avogadro's number, A_L the surface area per lipid molecule, and r the vesicle radius. Using $c_L = 10$ mM and $r = 50$ nm for the release experiments performed in the present study and $A_L = 0.68$ nm^2 for POPC,[27] this ratio amounts to 3.4% of the free detergent in the syringe which, for these examples, makes up $<1\%$ of the total SDS concentration (see Figure 5b in Results and Discussion).

Detergent translocation can then be assessed by comparing uptake and release experiments, which will yield a consistent set of thermodynamic parameters only for the correct choice of γ. The lipid accessibility factor of LUVs is expected to amount to $\gamma = 1.0$ if the detergent can equilibrate across the membrane or to $\gamma = 0.5$ if it cannot measurably overcome the bilayer within the time needed for an ITC run. Intermediate values stemming from partial translocation point to nonequilibrium states and must not be interpreted in terms of our model, which assumes that partitioning comes to a halt before the next injection is carried out. On one hand, membrane translocation in such a case must be so slow that the power signal associated with continued redistribution of the detergent between the aqueous phase and the membrane becomes indistinguishable from the baseline. On the other hand, this process cannot be as slow as to be completely negligible during the whole ITC experiment. This means that the heat integral under any given peak reflects binding to or desorption from the outer leaflet only, but the entire experiment is biased by an ongoing shift in the partition equilibrium because of gradual involvement of the inner leaflets of the vesicles injected in the beginning of the titration. Although there is no remedy to this problem, a combination of ITC uptake and release experiments may serve as an independent corroboration that the assumptions of the partitioning model used are indeed fulfilled.[28] For instance, incomplete equilibration in general or markedly composition-dependent partition coefficients and binding enthalpies would lead to erroneous results with a single uptake titration but can be detected by doing both uptake and release measurements.[15]

Electrostatic Effects. The partition equilibrium of an ionic detergent between the membrane and the bulk aqueous phase in terms of $K_D^{b/aq}$ is modulated by electrostatic repulsion from (or, in the case of oppositely charged lipid headgroups, attraction to) the membrane surface.[29] The partition equilibrium is then established between the bilayer and the interfacial (i) aqueous phase rather than the bulk solution and is described by an

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intrinsic partition coefficient defined as

$$K_D^{b/i} \equiv \frac{X_D^b}{X_D^i} \tag{10}$$

X_D^i denotes the mole fraction of detergent in the interfacial aqueous phase and is related to the corresponding bulk value, X_D^{aq}, by a Boltzmann term,

$$K_D^{i/aq} \equiv \frac{X_D^i}{X_D^{aq}} = \exp\left(\frac{-z_D e \Delta\varphi^{i/aq}}{kT}\right) \tag{11}$$

where z_D is the signed charge number of the detergent (here, $z_D = -1$), e the elementary charge, $\Delta\varphi^{i/aq}$ the electrostatic potential at the membrane surface with respect to the bulk aqueous phase, k the Boltzmann constant, and T the absolute temperature. Thus, the apparent partition coefficient reads

$$K_D^{b/aq} = K_D^{b/i} K_D^{i/aq} = K_D^{b/i} \exp\left(\frac{-z_D e \Delta\varphi^{i/aq}}{kT}\right) \tag{12}$$

Based on the Poisson equation and the Boltzmann distribution, Gouy−Chapman theory[30−32] relates $\Delta\varphi^{i/aq}$ to the membrane surface charge density, σ, according to

$$\sigma = \mathrm{sgn}(\Delta\varphi^{i/aq}) \sqrt{2000 RT \epsilon_0 \epsilon_r \sum_I c_I^{aq} \left(\exp\left(\frac{-z_I e \Delta\varphi^{i/aq}}{kT}\right) - 1\right)} \tag{13}$$

with R being the universal gas constant, ϵ_0 the permittivity of free space, and ϵ_r the dielectric constant of the medium, which, in the temperature range studied here, can be regarded as a linear function of temperature[33] according to $\epsilon_r = 174 - 0.32 \times T/\mathrm{K}$. The summation in eq 13 goes over the bulk aqueous concentrations, c_I^{aq}, of all ionic species (I), including the detergent, the buffer (here, 10 mM phosphate) and its counterions (16 mM Na^+), and the additional salt (154 mM NaCl), all of which contribute to the screening of the membrane potential. As above, $c_D^{aq} = c_D - c_D^b$ for the detergent; the other bulk concentrations may be approximated by the corresponding total concentrations, $c_I^{aq} = c_I$. The Henderson−Hasselbach equation provides the fraction of protonated buffer as $1/(1 + 10^{\mathrm{pH}-\mathrm{p}K_a})$, where p$K_a$ refers to the buffering group. In the present case, p$K_a = 7.2$ is the second pK_a value of phosphate, implying that 3.9 mM of the buffer carries a charge of $-e$, while the remaining 6.1 mM has a charge of $-2e$.

By definition, a second, independent expression[29] for the surface charge density is afforded by

$$\sigma = \frac{z_D e R_D^b}{A_L + R_D^b A_D} \tag{14}$$

where $R_D^b \equiv c_D^b / c_L$ is the detergent/lipid mole ratio in the bilayer, and A_D the molecular surface area requirement of membrane-incorporated detergent, which accounts for membrane expansion. Here, we chose $A_L = 0.68$ nm^2 for POPC[27] and $A_D = 0.30$ nm^2 for SDS.[24] Hence, $\Delta\varphi^{i/aq}$ is given implicitly by the equality of eqs 13 and 14 and can be calculated by standard iteration methods.

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Fitting Procedure. On the basis of ideal mixing, uptake and release titrations can be fitted with eqs 3 and 6−9. For a charged detergent, eq 12 has to be substituted into eq 6, and $\Delta\varphi^{i/aq}$ is obtained from eqs 13 and 14. We implemented this set of equations in an Excel (Microsoft, Redmond, U.S.A.) spreadsheet and performed nonlinear least-squares fitting with the Solver[34] add-in (Frontline Systems, Incline Village, U.S.A.) to find the values of $K_D^{b/i}$, $\Delta H_D^{b/aq}$, $Q_{L,dil}$, and $Q_{L+D,dil}$ that best describe the experimentally determined Q_L and Q_{L+D} data for a given value of γ. Alternatively, γ was also included as a floating parameter in the fitting procedure. The best-fit values obtained from independent experiments typically differed from each other by a factor <1.5 for $K_D^{b/i}$, by <1 kJ/mol for $\Delta H_D^{b/aq}$, and by <0.1 for γ. The first injection was always excluded from evaluation because it usually suffers from sample loss during the mounting of the syringe and the equilibration preceding the actual titration.

Results and Discussion

Half-Sided Membrane Binding at 25 °C. Figure 1a depicts ITC uptake and release raw data obtained at 25 °C for SDSand 100-nm-diameter POPC LUVs in 10 mM phosphate buffer, 154 mM NaCl, pH 7.4. The uptake experiment consisted of injecting 10-μL aliquots of 20 mM POPC to 100 μM SDS; the release experiment corresponded to diluting 10-μL aliquots of 10 mM POPC preloaded with 2 mM SDS into the 1.4-mL sample cell containing pure buffer. Under these conditions, reequilibration was so fast that time spacings of 5 min were sufficient for both uptake and release titrations, as can be seen from the prompt return of the differential heating power, Δp, to baseline level.

The corresponding heats of reaction, Q_L and Q_{L+D} (blue and red circles, respectively), in Figure 1b can be evaluated on the basis of ideal partitioning of SDS between the interfacial aqueous solution and the bilayer phase using eqs 3 and 6. Transbilayer distribution was taken into account by eqs 7−9, while electrostatic effects were implemented with the aid of eqs 12−14. Assuming no measurable flip−flop on the time scale of the experiment, which is quantified in terms of a lipid accessibility factor of $\gamma = 0.5$ (main panel), one obtains a good simultaneous fit (solid lines) to both data sets with an intrinsic mole fraction partition coefficient of $K_D^{b/i} = 3.5 \times 10^6$, a molar transfer enthalpy of $\Delta H_D^{b/aq} = -19.5$ kJ/mol, and dilution heats of $Q_{L,dil} = -0.03$ kJ/mol and $Q_{L+D,dil} = -0.07$ kJ/mol for uptake and release, respectively. In stark contrast to this, the presumption of swift transbilayer equilibration with $\gamma = 1.0$ fails to yield an acceptable fit (inset, $K_D^{b/i} = 1.6 \times 10^6$, $\Delta H_D^{b/aq} = -18.5$ kJ/mol, $Q_{L,dil} = -0.04$ kJ/mol, $Q_{L+D,dil} = -0.18$ kJ/mol). This is in agreement with radiolabel[2] and fluorescence[4] experiments; however, the present calorimetric approach not only abolishes the need for costly and laborious labeling of the detergent or the membrane but, in addition, is completed within a few hours only.

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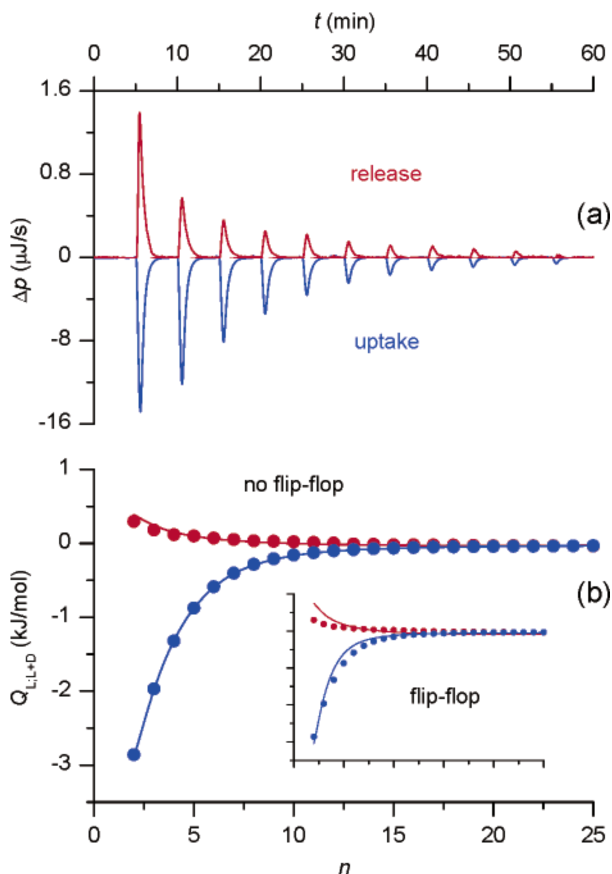

Figure 1. SDS uptake into and release from POPC LUVs at 25 °C. (a) Differential heating power, Δp, versus time, t. Uptake: 10-μL aliquots of 20 mM POPC were titrated to 100 μM SDS. Release: 10-μL aliquots of 10 mM POPC preloaded with 2 mM SDS were injected into buffer. Only 11 of 25 peaks are shown. (b) Normalized reaction heats, Q_L and Q_{L+D}, versus injection number, n. Experimental data (circles) and best global fits (solid lines) according to eqs 3, 6−9, and 12−14 for $\gamma = 0.5$ (main panel) and $\gamma = 1.0$ (inset). The first peak was not taken into account.

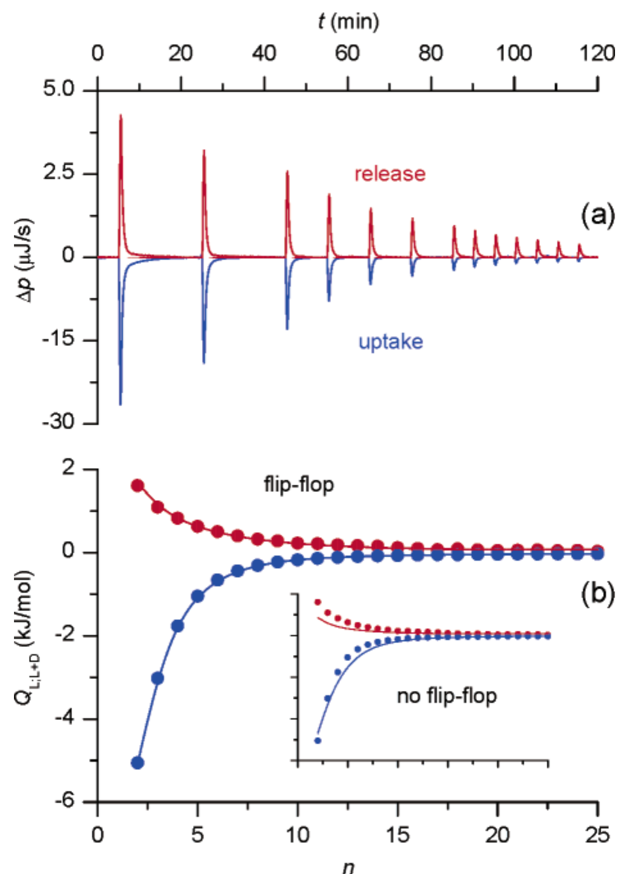

Figure 2. SDS uptake into and release from POPC LUVs at 65 °C. (a) Differential heating power, Δp, versus time, t. Other experimental conditions as in Figure 1. Only 13 of 25 peaks are shown. (b) Normalized reaction heats, Q_L and Q_{L+D}, versus injection number, n. Experimental data (circles) and best global fits (solid lines) according to eqs 3, 6−9, and 12−14 for $\gamma = 1.0$ (main panel) and $\gamma = 0.5$ (inset). The first peak was not taken into account.

Transmembrane Flip−Flop at 65 °C. A different picture emerged at 65 °C under otherwise identical conditions, as the calorimetric traces in Figure 2a demonstrate that, for the first two injections in both uptake and release experiments, time spacings of 20 min were necessary for complete equilibration. In Figure 2b, the integrated heats, Q_L and Q_{L+D} (blue and red circles, respectively), are fitted (solid lines) excellently using $\gamma = 1.0$, $K_D^{b/i} = 1.4 \times 10^6$, $\Delta H_D^{b/aq} = -31.8$ kJ/mol, $Q_{L,dil} = -0.02$ kJ/mol, and $Q_{L+D,dil} = -0.09$ kJ/mol (main panel). Here, imposing $\gamma = 0.5$ does not allow for a proper reproduction of the experimental data (inset, $K_D^{b/i} = 3.2 \times 10^6$, $\Delta H_D^{b/aq} = -32.8$ kJ/mol, $Q_{L,dil} = -0.01$ kJ/mol, and $Q_{L+D,dil} = 0$). Thus, the slower processes observed at elevated temperature in uptake and release titrations must be ascribed to delayed binding to and desorption from the inner leaflet, respectively. Interestingly, if γ were assumed to amount to the same value at both temperatures, the best-fit value of $K_D^{b/i}$ would only slightly decrease from 3.5×10^6 to 3.2×10^6 for $\gamma = 0.5$ or from 1.6×10^6 to 1.4×10^6 for $\gamma = 1.0$ upon going from 25 to 65 °C. However, such a weak temperature dependence of $K_D^{b/i}$ would not be reconcilable with pronouncedly exothermic $\Delta H_D^{b/aq}$ values as observed for the partitioning of SDS into POPC membranes (see next section).

Temperature Dependence. To draw a more detailed picture of SDS partitioning into POPC LUVs in the temperature range between the two limiting cases at 25 and 65 °C presented in Figures 1 and 2, respectively, we conducted uptake and release experiments also at 35, 45, and 55 °C. Table 1 summarizes the thermodynamic parameters obtained for the assumption of ideal mixing according to eq 4. In contrast to the extreme-case scenarios with a lipid accessibility factor fixed at $\gamma = 0.5$ or $\gamma = 1.0$, as shown in Figures 1 and 2, γ was allowed to vary during the fitting procedure here. Even so, the best-fit values of γ represent, within the experimental error limit of about 0.1, the two ideal cases of half-sided binding or complete trans-membrane equilibration at temperatures below and above 55 °C, respectively. By contrast, only intermediate values but

Table 1. Thermodynamics of SDS Partitioning between the Aqueous Phase and POPC LUVs on the Basis of Ideal Mixing According to Eq 4 as a Function of Temperature, T^a

T °C	$K_D^{b/i}$ (10^6)	$\Delta G_D^{b/i,0}$ (kJ/mol)	$\Delta H_D^{b/aq}$ (kJ/mol)	$-T\Delta S_D^{b/i,0}$ (kJ/mol)	γ
25	4.5	−38.0	−19.6	−18.4	0.5
35	3.3	−38.5	−22.3	−16.2	0.5
45	2.5	−39.0	−25.5	−13.5	0.6
65	1.4	−39.8	−31.9	−7.9	1.0

^{a} Mole fraction partition coefficients, $K_D^{b/i}$, molar transfer enthalpies, $\Delta H_D^{b/aq}$, and accessibility factors, γ, were determined directly from uptake and release experiments using eqs 3, 6−9, and 12−14. Standard molar Gibbs free energies, $\Delta G_D^{b/i,0}$, and entropic terms, $-T\Delta S_D^{b/i,0}$, were calculated using eqs 15 and 16, respectively. See the following section for a discussion of experiments performed at 55 °C.

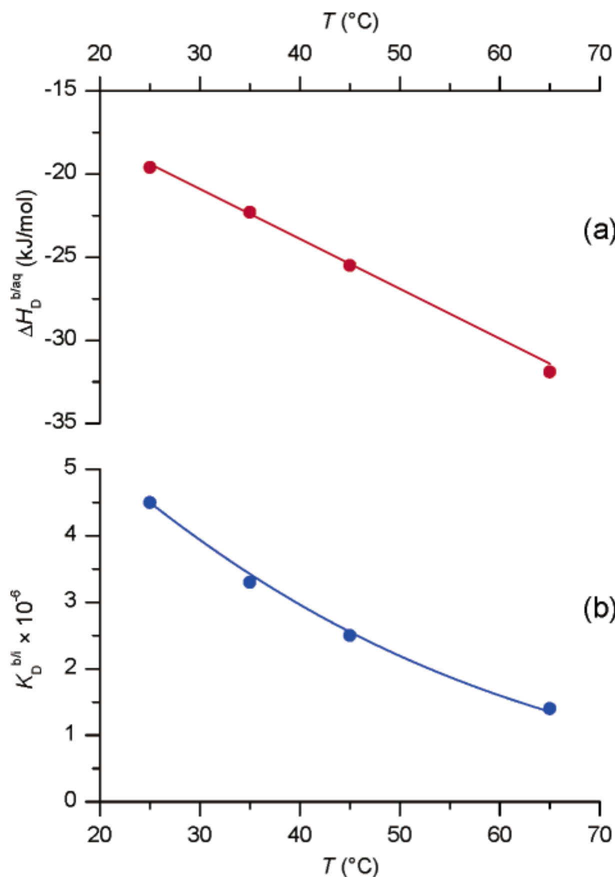

Figure 3. Thermodynamics of SDS membrane partitioning as a function of temperature, T. (a) Molar transfer enthalpy from the aqueous into the bilayer phase, $\Delta H_D^{b/aq}$. (b) Intrinsic mole fraction partition coefficient, $K_D^{b/i}$. Experimental data (circles; see Table 1 for numerical values) and best fits (solid lines) according to eqs 17(a) and 18(b). See the following section for a discussion of experiments performed at 55 °C.

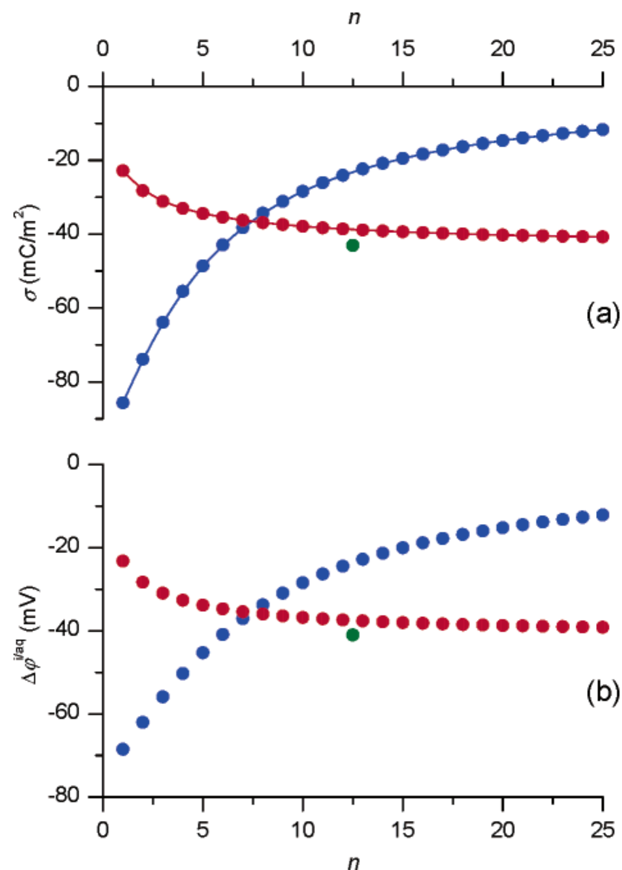

Figure 4. Membrane electrostatics of SDS binding to POPC LUVs at 25 °C. (a) Membrane surface charge density, σ, as given by eq 13 (lines) and eq 14 (circles). (b) Electrostatic membrane potential, $\Delta\varphi^{i/aq}$, as calculated from eqs 13 and 14. n is the injection number. Cell content during uptake (blue) and release (red) titrations and syringe content during the release (green) experiment shown in Figure 1.

neither $\gamma = 0.5$ nor $\gamma = 1.0$ can account for the results obtained at 55 °C (data not shown, see next section). Note that the SDS concentration was always much below the critical micellar concentration (CMC), which increases from 0.91 mM at 25 °C to 1.63 mM at 65 °C under the conditions used here.[22]

The standard molar Gibbs free energy of SDS transfer from the interfacial aqueous phase into the membrane is

$$\Delta G_D^{b/i,0} = -RT \ln K_D^{b/i} \quad (15)$$

Using the approximation $\Delta H_D^{b/aq} = \Delta H_D^{b/i} = \Delta H_D^{b/i,0}$, the Gibbs−Helmholtz equation yields the entropic contribution as

$$-T\Delta S_D^{b/i,0} = \Delta G_D^{b/i,0} - \Delta H_D^{b/i,0} \quad (16)$$

with $\Delta S_D^{b/i,0}$ denoting the standard molar transfer entropy.

Figure 3a illustrates the temperature dependence of the molar transfer enthalpy, $\Delta H_D^{b/aq}$. In the temperature range studied here, the relationship between $\Delta H_D^{b/aq}$ and T can be described by

$$\Delta H_D^{b/aq}(T) = \Delta C_{p,D}^{b/aq}(T - T_0) \quad (17)$$

where $\Delta C_{p,D}^{b/aq}$ is the change in molar heat capacity at constant pressure, p, and T_0 the extrapolated temperature at which $\Delta H_D^{b/aq}(T_0) = 0$. The best fit (solid line) to the experimental data (circles) gives $\Delta C_{p,D}^{b/aq} = -0.30$ kJ/(mol K) and $T_0 = -40$ °C. Figure 3b depicts the van't Hoff prediction (solid line) of the temperature dependence of the intrinsic partition coefficient, $K_D^{b/i}$, according to

$$K_D^{b/i}(T) = K_D^{b/i}(T_0) \exp\left(-\frac{\Delta C_{p,D}^{b/aq}}{R}\left(1 - \frac{T_0}{T} + \ln\frac{T_0}{T}\right)\right) \quad (18)$$

which describes the experimental data (circles) very well using $K_D^{b/i}(T_0) = 1.2 \times 10^7$ and the values of T_0 and $\Delta C_{p,D}^{b/aq}$ derived above from eq 17.

Intermediate Values of γ. The best fit to the data collected at 55 °C gives $\gamma = 0.7$, which deviates significantly from the only two thermodynamically meaningful cases characterized by $\gamma = 0.5$ or $\gamma = 1.0$ for half-sided binding and rapid transmembrane equilibration, respectively. As mentioned in the Theory, intermediate γ values reflect flip−flop rates that do not permit a rigorous thermodynamic treatment. Importantly, the time spacings between consecutive injections were always chosen long enough to allow for return of the ITC signal, Δp, to baseline level, as judged by visual inspection of the raw data (cf. Figures 1a and 2a). At 55 °C, the initial spacings amounted to 20 min; reducing them to 15 min or less led to lower values of γ, whereas considerably but arbitrarily extending them beyond 20 min gave rise to somewhat higher values. This means that, at the temperature in question, SDS flip−flop across the membranes of 100-nm POPC vesicles is clearly detectable but not completed within 20 min. Beyond this time point, however, translocation becomes too slow to produce a discernible ITC

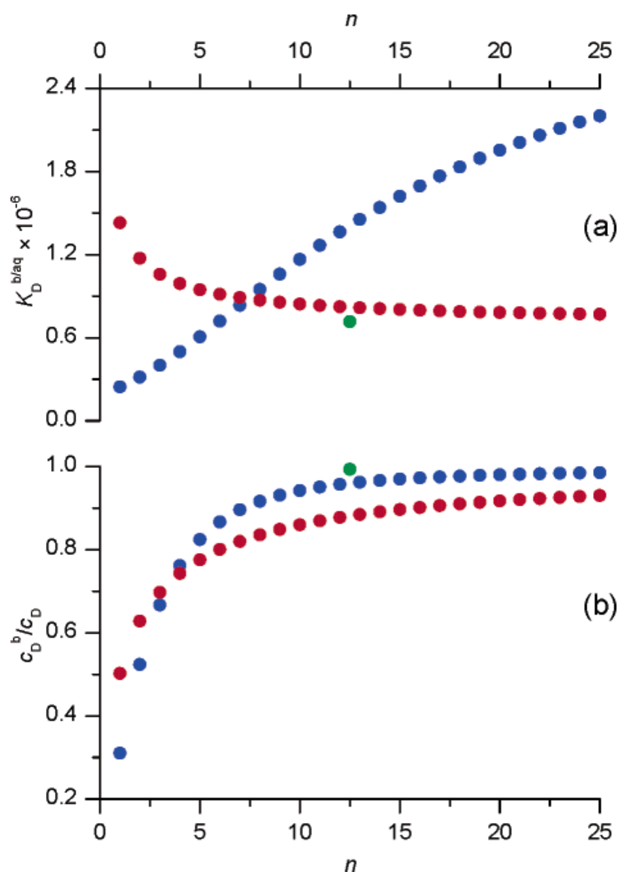

Figure 5. Parameters characterizing SDS binding to POPC LUVs at 25 °C. (a) Apparent mole fraction partition coefficient, $K_D^{b/aq}$, as defined by eq 4 and given by eq 12. (b) Fraction of membrane-bound detergent, c_D^b/c_D, as obtained from eq 6 and the total detergent concentration, c_D. *n* is the injection number. Cell content during uptake (blue) and release (red) titrations and syringe content during the release (green) experiment shown in Figure 1.

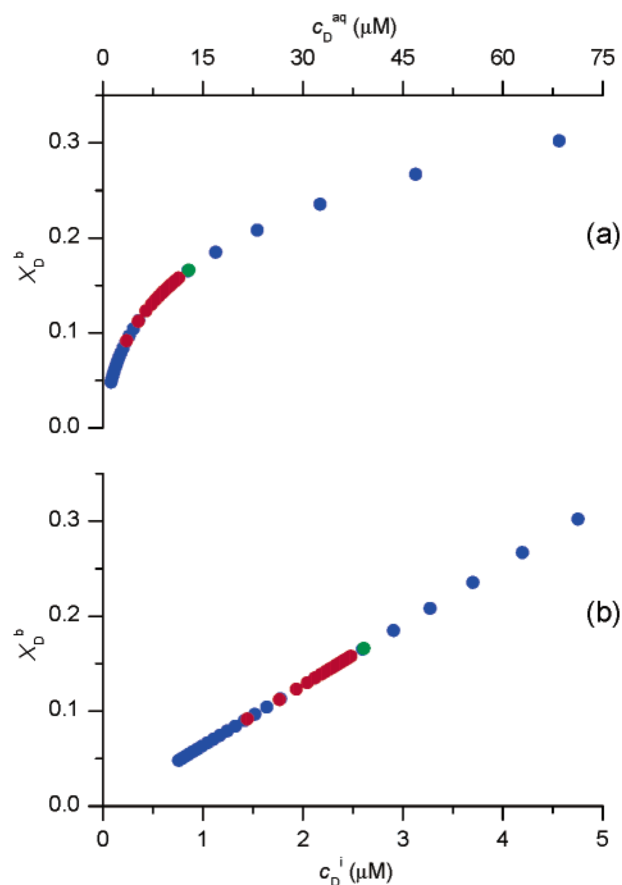

Figure 6. Partitioning of SDS between the aqueous phase and POPC LUVs at 25 °C. (a) Mole fraction of detergent in the membrane, X_D^b, versus bulk aqueous detergent concentration, c_D^{aq}. This represents the partition equilibrium described by eq 4. (b) X_D^b versus interfacial aqueous detergent concentration, c_D^i, representing the partition equilibrium given by eq 10. Cell content during uptake (blue) and release (red) titrations and syringe content during the release (green) experiment shown in Figure 1.

signal. This is a kinetic phenomenon and, as such, is not amenable to assays based on equilibrium thermodynamics, such as the one discussed here. Apart from temperature, a variety of experimental parameters are expected to affect the kinetics of SDS flip−flop, for example, membrane curvature (i.e., vesicle size), lipid composition (e.g., degree of acyl chain saturation), ionic strength, and detergent content in the membrane. The last parameter is particularly important but, obviously, cannot be kept constant in titration experiments.

In view of this, it is intriguing that values of $K_D^{b/i} = 2.0 \times 10^6$ and $\Delta H_D^{b/aq} = -27.9$ kJ/mol calculated for $\gamma = 0.7$ at 55 °C would almost perfectly obey the temperature dependences illustrated in Figure 3. Nevertheless, this appears to be a fortuitous coincidence that should not overshadow the fact that intermediate γ values lack a thermodynamically valid meaning and must not be interpreted quantitatively. Instead, they reflect incomplete equilibration on the experimental time scale, and the asset of combining ITC uptake and release assays lies in the straightforward identification of such kinetically controlled systems.

Key Parameters. In addition to the theoretical values of the normalized reaction heats, Q_L and Q_{L+D}, the equations derived in the Theory supply a wealth of information on the thermodynamics and electrostatics of SDS partitioning into POPC membranes. Some key parameters derived from the experiments at 25 °C shown in Figure 1 are briefly surveyed in the following. Note that the values for the content of the calorimeter cell refer only to the accessible detergent and lipid, whereas the values for the syringe content in the release protocol apply to both leaflets.

Figure 4a provides a summary of membrane electrostatics in terms of the surface charge density, σ. The equality of the σ values calculated with eq 13 (lines) and those obtained with eq 14 (circles) allows for calculation of $\Delta\varphi^{i/aq}$, which, as shown in Figure 4b, amounts to considerable values and varies substantially during both experiments.

Figure 5a shows the apparent mole fraction partition coefficient, $K_D^{b/aq}$, which is afforded by eq 12. Owing to the alterations in membrane electrostatics discussed above, $K_D^{b/aq}$ increases by a factor of 10 or decreases by a factor of 2 during uptake and release, respectively, thus underlining the necessity of properly accounting for electrostatic forces at the membrane surface. Figure 5b gives the fraction of detergent that is bound to the membrane, c_D^b/c_D, as obtained from eq 6. c_D^b/c_D exceeds 98 or 93% toward the end of uptake and release experiments, respectively. The fact that $c_D^{b,s}/c_D^s$ in the syringe is even greater than 99% during the release titration supports the validity of eq 8, which neglects the fraction of detergent entrapped in the intravesicular aqueous solution.

Figure 6a depicts the mole fraction of detergent in the membrane, X_D^b, as a function of the bulk aqueous detergent

concentration, c_D^{aq}. Whereas this curve is bent downward with increasing c_D^{aq}, plotting X_D^b versus the interfacial aqueous detergent concentration, c_D^i, as in Figure 6b, yields a straight line with a slope of $K_D^{b/i}/c_W$. This exemplifies the suitability of combining a surface partition equilibrium with simple electrostatic theory to describe the binding behavior of an ionic detergent to a lipid membrane.

Outlook

The temperature dependence of the flip−flop process entails major consequences for the solubilization of lipid membranes and their proteinaceous constituents, as SDS exhibits a behavior reminiscent of that of nonionic detergents at 56 °C[24] and 65 °C[22] but not at ambient temperature.[2,4,22] Thus, bilayer permeation plays an eminent role in biochemical procedures involving surfactants, such as membrane dissolution, detergent extraction, and membrane protein crystallization. For instance, a nonpermeant detergent can adversely affect the reconstitution of cellular membrane components[2] because it is hardly extractable from the inner leaflet of reconstituted liposomes. Moreover, fast membrane flip−flop has also been inferred to be a key prerequisite for several clinical applications of detergents, such as contraception and the prevention of sexually transmitted diseases.[35]

The ITC protocol delineated here for the example of the anionic detergent SDS has already been applied successfully to diverse other compounds intended for various purposes. First, Apel-Paz et al.[35] have employed it for the characterization and comparison of ionic and nonionic surfactants as drugs or drug candidates, including SDS. Second, Cambridge and co-workers[36,37] have exploited ITC to ensure that a caged doxycycline derivative developed for photoactivated gene expression can indeed reach its intranuclear target by transmembrane diffusion upon extracellular administration. Third, we have utilized uptake and release experiments to probe bilayer translocation of penetratin,[38] a cell-penetrating peptide hypothesized to traverse membranes by a yet elusive mechanism.[21]

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Conclusions

In conclusion, we present for the first time the use of ITC for assessing the flip−flop of charged compounds across a lipid membrane. The capability of overcoming such a barrier is a critical determinant of the function and applicability of a wide array of bioactive molecules, and a universal and straightforward assay for following their transbilayer distribution hence appears valuable. Beyond membrane biophysics, this approach may therefore be beneficial for numerous aspects in the fields of medicinal chemistry, cell biology, and drug development.

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Supporting Information Available: Experimental procedures and alternative partitioning model. This material is available free of charge via the Internet at http://pubs.acs.org.

JA056389D

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