ANALYSIS OF MEMBRANE PROTEIN DIMERIZATION BY SEDIMENTATION EQUILIBRIUM*

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Abstract

Determination of the assembly state(s) of a membrane protein poses a difficult analytical problem. A powerful technique that is well adapted to meet this challenge is solution interaction analysis using analytical ultracentrifugation. Due to their hydrophobic nature, membrane proteins require detergent (or lipid) for solubilization in solution. Thus, a fundamental obstacle to be overcome in determining the stoichiometry of membrane proteins is that the mass of any bound detergent molecules will contribute to the experimentally determined apparent molecular weight. Judicious choice of detergent and solvent conditions surmounts this obstacle and facilitates evaluation of the mass of *only* the protein component in a protein–detergent complex. Employing this experimental strategy, sedimentation equilibrium approaches were used to determine the association states of the glycophorin A transmembrane (GpATM) dimer in solutions of two detergents, $C_{12}E_8$ and C_8E_5. Analysis of sedimentation equilibrium data collected in C_8E_5 micelles allowed estimation of a monomer/dimer equilibrium constant from which the free energy of dimerization could be calculated. Equilibrium constants were also determined for GpATM mutants that had previously been shown in a qualitative manner to diminish dimerization. Thus, the earlier mutational sensitivity results are placed on a quantitative, relative scale of interaction by ultracentrifuge experiments. This bulletin will focus on explaining the theoretical and practical considerations for making these types of experimental measurements on membrane proteins.

Introduction

Integral membrane proteins can often be isolated and solubilized in a functional form by the use of detergents. As with soluble proteins, an understanding of the sequence/structure/function relationships of a membrane protein begins with a determination of the molecular weight(s) and stoichiometry of the protein components required for functional activity. Unfortunately, commonly used techniques for estimating molecular weight are problematic for membrane proteins due to the necessity of detergents for solubilization and activity. While SDS-PAGE is a useful technique for obtaining molecular weight estimates of protein components, it is not uncommon for membrane proteins to migrate anomalously on SDS-PAGE.[1] Gel filtration and other hydrodynamic techniques are not favorable since the experimentally measured hydrodynamic radius can be dominated by the hydrodynamic properties of the micelle, leading to overestimations of the protein molecular weight. Finally, due to detergent contributions to the scattering signal, multi-angle light scattering data cannot be simply and automatically interpreted for detergent-solubilized proteins in a manner analogous to that done for soluble proteins.

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Sedimentation equilibrium analysis can overcome many of these problems. Sedimentation equilibrium has long been recognized as the technique of choice for analysis of solution interactions of macromolecules.[2]

While the determination of a membrane protein molecular weight formally requires knowledge of the amount of detergent bound, experimental approaches have demonstrated that, in practice, the contribution of detergent can be minimized and the molecular weight of the protein *alone* can be obtained. Sedimentation equilibrium can be carried out in a wide variety of detergent environments, over a range of pH values, temperatures and ionic strengths. It is a nonperturbing technique, requires no standards for calibration, and does not necessitate labeling of the protein as long as the detergent of choice does not absorb at the wavelength of interest. Thus, the molecular weight of a reconstituted membrane protein can be rigorously analyzed under a wide variety of solubilization conditions.

We have used sedimentation equilibrium to evaluate the solution molecular weights and association energy of the glycophorin A transmembrane dimer. A paradigm for the two-stage model of membrane protein folding, glycophorin A has been shown to form homodimers through self-association of its single, helical transmembrane domain.[1,3-6] The sequence dependence of dimerization has been elucidated by Engelman and coworkers using an SD-SPAGE assay.[1,5] The structure of the transmembrane dimer in dodecylphosphocholine micelles has been solved,[7] thereby providing a three-dimensional framework for interpretation of the dimerization mutational sensitivity. While the mutational sensitivity as measured by SDS-PAGE qualitatively describes the effects of the point mutations, this manuscript summarizes how sedimentation equilibrium has allowed a quantitative evaluation of the effects of mutations on the free energy of dimerization.[8,9]

Materials and Methods

The *Staphylococcus* nuclease-GpA transmembrane domain fusion protein (SN/GpATM) was expressed and purified from *Escherichia coli as previously* described in detail.[5] Prior to sedimentation equilibrium analysis, the protein was exchanged into *either* $C_{12}E_8$ or C_8E_5 by adsorption to an ion exchange column, washing with 10 volumes of buffer containing the appropriate detergent, and eluting with high salt. Samples were then adjusted to contain the proper buffer concentrations by either dilution or dialysis.

Sedimentation equilibrium experiments were conducted using the absorbance optics of a Proteomelab XL-I analytical ultracentrifuge from Beckman Coulter. The protein was run at three initial protein concentrations (0.3, 0.5 and 0.8 OD/mL at 230 nm) in 20 mM sodium phosphate, pH 7.0, containing 200 mM NaCl supplemented with the appropriate detergent. Sample volumes of 110 μL were used in six-sector cells equipped with quartz windows. All experiments were conducted at 25°C, and sedimentation equilibrium data were collected at 20000, 24500, and 30000 rpm. The partial specific volume of the protein was calculated as 0.7476 mL g^{-1} using the values of Cohn & Edsall.[10] The program Sednterp was used to calculate the solvent densities from the buffer components.[11] For experiments in $C_{12}E_8$, a solvent density of 1.027 g mL^{-1} was obtained by the addition of 18% v/v D_2O. For experiments in C_8E_5, no addition of D_2O was necessary and the solvent density was calculated as 1.0075 g mL^{-1}. Sedimentation equilibrium data were evaluated using the Windows 95 version of the nonlinear least-squares curve-fitting algorithm, NONLIN.[12]

Theoretical Considerations

The molecular weight of a protein in a detergent or lipid solution can be determined unambiguously if the contribution of the detergent or lipid to the sedimenting particle is treated appropriately. Since membrane proteins, by their nature, require detergent micelles or lipid vesicles in order to be soluble in solution, the buoyant mass of the sedimenting particle in the ultracentrifuge, $M_{Pr}(1-\phi'\rho)$, will contain contributions from both the protein as well as the detergent or lipid (where M_{Pr} is the mass of the protein alone, ϕ' is the effective partial specific volume of the protein–detergent complex, and ρ is the solvent density). In order to account for these separate contributions, the buoyant molecular weight of the complex can be rewritten in terms of its components.[13,14]

$$M_{Pr}(1-\phi'\rho) = M_{Pr}[(1-\bar{v}_{Pr}\rho) + \delta_{Det}(1-\bar{v}_{Det}\rho)]$$

1

where $\bar{v}_{Pr}$ and $\bar{v}_{Det}$ are the partial specific volumes of the protein and detergent, respectively, and δ_{Det} is the amount of detergent bound in grams per gram of protein.

If the amount of bound detergent and its partial specific volume are known, then these values can be substituted into Eq. (1) in order to solve for the protein molecular weight. Often, however, the investigator is lacking one or both pieces of information. In this case, the contribution of the detergent can be experimentally minimized by matching the density of the detergent micelle with the solvent buffer such that $\rho = 1/\bar{v}_{Det}$. The value of the term $\delta_{Det}(1-\bar{v}_{Det}\rho)$ in Eq. (1) then approaches zero, and the analysis of this multicomponent system becomes pseudo-two component with $M_{Pr}(1\phi'\rho)$ essentially equal to $M_{Pr}(1-\bar{v}_{Pr}\rho)$. Figure 1 shows a schematic of this density matching strategy. It is preferable to match the micelle density with heavy water as opposed to sucrose, since this should minimally perturb the water activity.[15] Thus, detergents that are the most experimentally favorable will have micelle partial specific volumes in the range of 0.9 to 1.0. Extensive compilations of data for detergent and phospholipid partial specific volumes can be found in Steele, *et al.*,[16] and in Durshlag.[17]
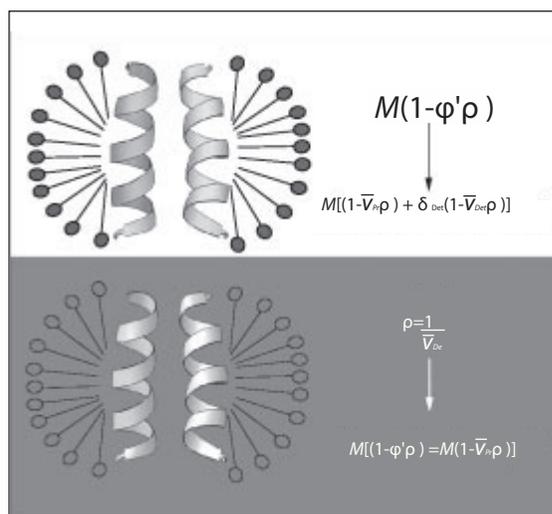
Figure 1. Schematic depicting density matching strategy. Adjustment of the solvent density to approach that of the bound detergent results in minimimization of the detergent contribution to the experimentally measured buoyant mass.

When it is not technically possible to match the detergent with the solvent density, experiments can be done at several H_2O/D_2O ratios. The buoyant molecular weight of the sedimenting particle can then be determined by extrapolation outside of the experimental range. An example of this strategy applied to analysis of apolipoprotein A can be found in Reynolds & McCaslin.[15] While this extrapolation procedure is not desirable, since it is accompanied by a loss of precision, membrane protein complexes requiring bile salt detergents for solubilization or activity must be analyzed in this manner due to the low partial specific volumes of the bile salt detergents.[16]

Results and Discussion

*Molecular Weight Distribution of SN/GpATM in $C_{12}E_8$*

The solution interactions of the SN/GpATM dimer were first evaluated in $C_{12}E_8$ detergent solutions. $C_{12}E_8$ is a ''nondenaturing,'' nonionic polyoxyethylene ether detergent with a long history of use in the ultracentrifuge for determination of membrane protein molecular weights.[14,15,18] Technically, density matching $C_{12}E_8$ micelles is feasible, since the micelle partial specific volume is reported to be 0.973 mL g^{-1}.[16] At 20°C in buffer containing 20 mM sodium phosphate and 200 mM sodium chloride, the density can be matched with 18% D_2O.

Typical sedimentation equilibrium data for SN/GpATM in $C_{12}E_8$ are shown in Figure 2. The simplest model that was found to describe the radial distributions indicates that SN/GpATM forms three oligomeric species in $C_{12}E_8$: monomer, dimer and tetramer. The formal expression is given below:

$$c_i = c_{ref}\, exp[\, \acute{O}\, (\, \xi_i - \xi_{ref}\,)] + c^2_{ref} K_{1,2}\, exp[2\, \acute{O}(\xi_i - \xi_{ref}\,)] + c^4_{ref} K_{1,4}\, exp[4\, \acute{O}(\, \xi_i - \xi_{ref}\,)] + base \qquad \textbf{2}$$

where c_i is the total absorbance at a radial position, r_i; c_{ref} is the monomer absorbance at a reference position, r_{ref}; $Ó= M(1 - \bar{v}\rho)\omega^2/RT$; M is the monomer molecular mass; $\bar{v}_{Pr}$ is the monomer partial specific volume; ρ is the solvent density; ω is the angular velocity (radians sec^{-1}); R is the universal gas constant; T is the absolute temperature; $\xi = r^2/2$; $K_{1,2}$ is the apparent monomer–dimer equilibrium constant, $K_{1,4}$ is the apparent monomer–tetramer equilibrium constant; and *base* is a baseline term for nonsedimenting material.
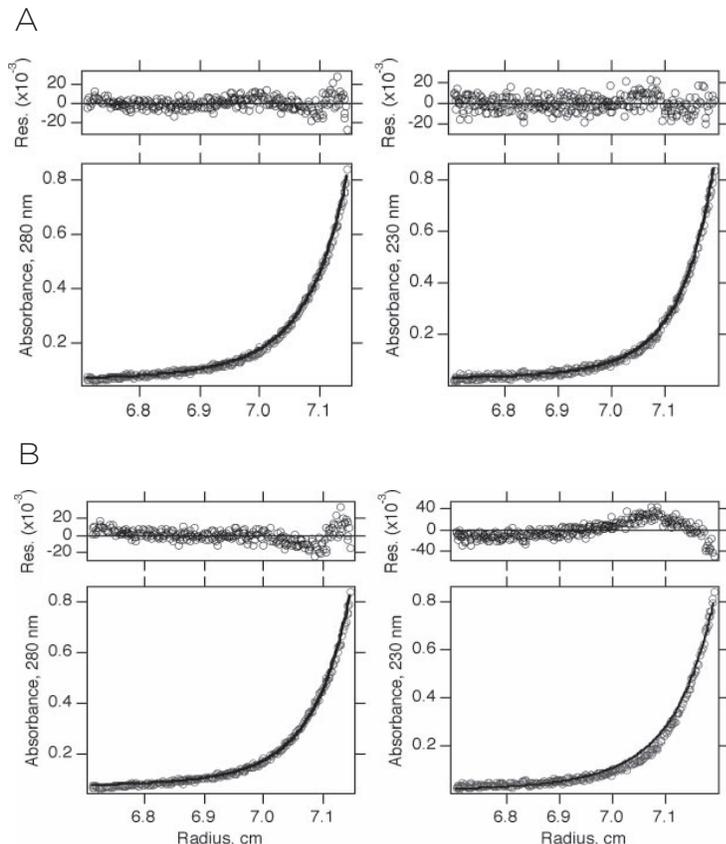
Figure 2. Analysis of SN/GpATM in $C_{12}E_8$. Sedimentation equilibrium data collected at *24500* rpm are shown for two initial concentrations (*32 μM*, left side and *2 μM*, right side). Panel *A* shows the results of fitting to Eq. (2) using local $K_{1,2}$ values. Panel *B* shows the results of fitting to Eq. (2) using a global $K_{1,2}$ value. The open circles are the data points and the solid line is the appropriate fit of Eq. (2) to the data. The residuals of the fit for each data set are shown above each radial distribution. Adapted from Fleming[8] with permission

Importance of the Global Fit in Estimating Equilibrium Constants

A major goal of solution interaction analysis of SN/GpATM was determination of helix/helix interaction energies. Obtaining an estimate for an equilibrium constant of an interacting system requires that the species in question reversibly associate with each other on the time scale of the experiment. In analysis of sedimentation equilibrium data, such reversibility will be reflected as a constant value for the equilibrium constant, *K,* over all initial concentrations and rotor speeds. Reversibility of interacting species is verified during the global fitting procedure in NONLIN by treating *K* as a global parameter (*i.e.*, a single value for *K* for all of the data sets). When species are *not* reversibly associating with each other on the time scale of the experiment, the use of a global *K* in the fitting function will result in a poor fit to the data as evidenced by nonrandomness of the residuals and poor fit statistics. The contrast between the use of a local versus a global $K_{1,2}$ parameter is shown in Figure 2 for two initial concentrations of SN/GpATM. While the data at each concentration are well described by Eq. (2) using local $K_{1,2}$ values (top panels), forcing the fitting function to use a global $K_{1,2}$ results in a poor fit (bottom panels). The fitting procedure thus suggests that the system is kinetically frozen on the time scale of the experiment, and it is not possible to obtain estimates for thermodynamic equilibrium constants using this technique. However, verification of the model (i.e., the oligomeric identities) is still possible by treating the *K* values as local parameters (*i.e.*, a separate *K* for each data set) as long as such a fitting function describes

the data with acceptable goodness of fit. Switching between models that use local and global K values is easily carried out in NONLIN.[12,19] Thus, although the oligomeric species are well defined in $C_{12}E_8$, estimation of the equilibrium constant in this micelle environment, at least for this protein, was not possible.

Molecular Weight Distribution of SN/GpATM in C_8E_5: Determination of Interaction Energetics

The distribution of SN/GpATM species was also evaluated in detergent solutions of C_8E_5. This detergent is chemically similar to $C_{12}E_8$ in that it is also a nonionic polyoxyethylene. The partial specific volume has been estimated as 0.993 g mL^{-1}.[20] Thus, it has the technical advantage that the micelles are neutrally buoyant at 25°C in buffer containing 20 mM sodium phosphate, 200 mM NaCl. Moreover, global fitting of sedimentation equilibrium data collected in C_8E_5 using Eq. (2) employing a global $K_{1,2}$ parameter was successful, indicating estimation of the equilibrium constant would be possible. The distribution of species at 30000 rpm is shown in Figure 3 where it can be seen that SN/GpATM is predominantly dimeric in 33 mM C_8E_5 at µM concentrations. The dimerization dissociation constant was estimated from the global fit to be 240 (±50) nM, corresponding to a dissociation free energy of 9.0 (±0.1) kcal mol^{-1}. Thus, SN/GpATM dimerizes with high affinity in the neutrally buoyant detergent C_8E_5.

Disruptive Mutants Reduce the Interaction Free Energy

Extensive mutagenesis of the glycophorin A transmembrane domain has been conducted using an SDS-PAGE assay. A striking outcome of the SDS-PAGE mutational sensitivity was the observation that even subtle point mutations at specific positions along the helix could significantly compromise dimerization.[1,5] With a protocol to rigorously measure interaction energies of transmembrane α-helices now in hand, point mutants at two positions in the GpATM were analyzed for their ability to dimerize in C_8E_5. The mutations Leu^{75}Ala and Ile^{76}Ala had previously been shown to significantly and completely (respectively) reduce dimerization, as measured by SDS-PAGE. $K_{2,1}$ estimates of 1.4 (±0.2) and 4.2 (±0.9) µM were obtained for these mutants, respectively, using sedimentation equilibrium in C_8E_5 at 25°C. The differences in the free energies between the wild type and the Leu^{75}Ala and Ile^{76}Ala mutants, respectively, are 1.1 (±0.1) and 1.7 (±0.2) kcal mol^{-1}. The distribution of the dimeric species for these mutants compared to the wild type sequence is shown in Figure 4. This presentation also illustrates the large concentration range wherein solution interaction analysis using analytical ultracentrifugation was carried out (the solid portions of the curves). Nearly two logs of concentrations were actually assayed in the sedimentation equilibrium experiment, and each global fit was conducted with approximately 1,000 primary data points. Thus, sedimentation equilibrium provides a powerful and quantitative method for analyzing the effect of mutations on the equilibrium of an associating system.
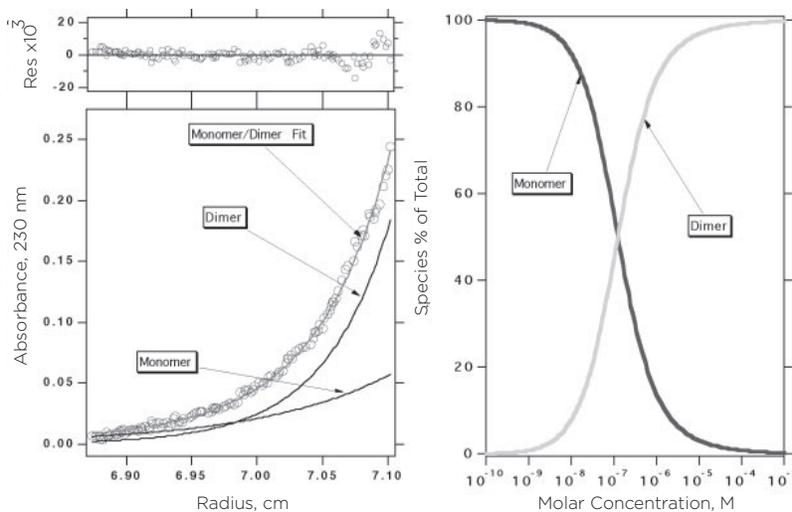
Figure 3. Analysis of SN/GpATM in C_8E_5. The distribution of species at 30,000 rpm is shown. The open circles are the data points and the solid line is the global fit of Eq. (2) to the data as described in the text. The dotted lines are the distribution of the monomer and dimer species whose sum gives rise to the observed fit. No tetramer is observed at this initial concentration and speed. The residuals of the fit are shown in the uppermost left panel. The right panel shows the distribution of the reversibly associating monomer and dimer species as a function of total protein concentration as calculated from the estimated equilibrium constant.

Summary and Conclusions

Sedimentation equilibrium experiments provide quantitative information about membrane proteins not otherwise available using analytical techniques. As long as conditions of reversibility are met, the free energy of interaction can be measured in varied hydrophobic environments, pH values, ionic strengths and temperatures. While the absolute value of the interaction free energy of membrane protein subunits will no doubt depend on the hydrophobic environment, experiments in C_8E_5 allow subunit associations to be placed on a relative scale of interaction. The temperature dependence of the free energy provides more detailed information on the thermodynamics of helix-helix association in micelles. The protocols reviewed here should be widely applicable to the analysis of macromolecular associations of membrane proteins in detergent solutions
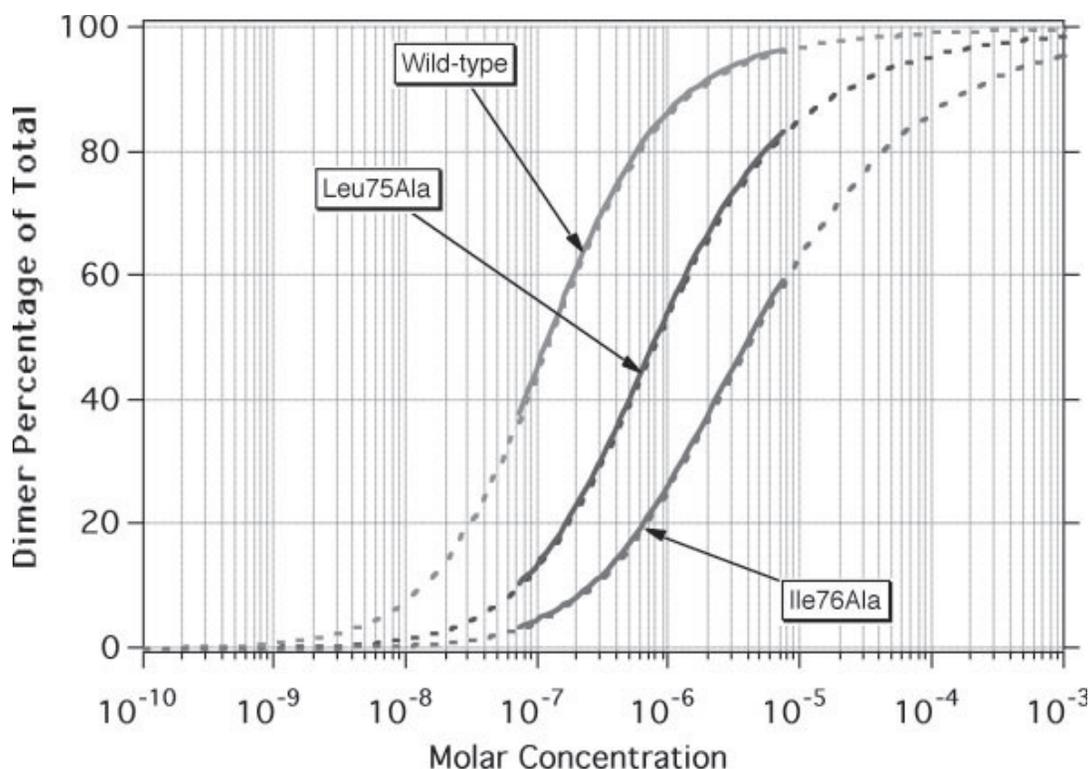
Figure 4. Species distribution of SNGpATM dimers in C_8E_5. The distribution of species was calculated from the monomer/dimer equilibrium constants determined from global fitting of sedimentation equilibrium data for each mutant. The solid lines are the concentration range wherein the analysis was carried out. The dotted lines are extrapolations of those data. Adapted from Fleming, et al.,[9] with permission.

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