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ELSEVIER

Biophysical Chemistry 108 (2004) 43–49

Biophysical Chemistry

www.elsevier.com/locate/bpc

Thermodynamics of glycophorin A transmembrane helix dimerization in C14 betaine micelles

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Abstract

We have used sedimentation equilibrium analytical ultracentrifugation to measure the free energy change for the glycophorin A transmembrane helix–helix dimerization in C14 betaine micelles. By varying the amount of micellar C14 betaine, we show that the protein association reaction in the micellar C14 phase behaves as an ideal-dilute solution. In this hydrophobic environment, the mole-fraction standard state free energy change for self-association of the SNGpA99 glycophorin A construct is -5.7 (± 0.3, $N=5$) kcal mol^{-1} at 25 °C. Compared with previous results carried out in C_8E_5 micellar solutions, the free energy of dimerization is 1.3 kcal mol^{-1} less favorable in C14 betaine micelles. In contrast, when considered on a per-interface basis, the formation of the glycophorin A transmembrane dimer in C14 betaine micelles may be more favorable than the association of several designed transmembrane peptides.

Keywords: Membrane protein; Transmembrane interactions; Interaction thermodynamics

1. Introduction

A fundamental question in the study of membrane proteins is how the polypeptide sequence specifies the native fold. For helical membrane proteins, a useful thermodynamic framework for folding suggests that transmembrane helix formation can be thermodynamically uncoupled from the subsequent side-to-side interactions that form the bundle of helices defining the native fold [1,2]. A question of active investigation is: What are the determinates for these side-to-side interactions? At a minimum the association of transmembrane α-helices will involve changes in protein–protein interactions, changes in lipid–lipid interactions and changes in protein–lipid interactions. Understanding the contribution from each of these reactions is an essential component of knowing how and why transmembrane helices interact.

Sedimentation equilibrium analytical ultracentrifugation has been instrumental in providing measurements of the energetics of transmembrane

Abbreviations: SNGpA99, a fusion protein composed of *Staphylococcal* nuclease and the transmembrane domain of human glycophorin A terminating at position 99 in the glycophorin A native sequence; SNGpA101, a fusion protein composed of *Staphylococcal* nuclease and the transmembrane domain of human glycophorin A terminating at position 101 in the glycophorin A native sequence; GpA, glycophorin A.

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doi:10.1016/S0301-4622(03)00296-5

helix–helix interactions in detergent micelle environments [3–12]. In contrast to spectroscopic techniques, the advantage of using sedimentation equilibrium for thermodynamic analysis is that it provides a direct measure of the molecular weight(s) of species in solution. In addition, the ability to experimentally measure the protein over a wide concentration range directly reports on the concentration dependence of the molecular weight(s), which yields the thermodynamic parameters for association. Using sedimentation equilibrium, we and others have determined the stoichiometry and energetics for both native and designed transmembrane sequences [3,6,7,9, 13,14]. However, one of the challenges faced in comparing results between studies on different proteins or in different hydrophobic environments has been the lack of a clear mechanism to calculate a standard state free energy change for protein association in the micellar environment. We have recently made progress towards overcoming this challenge by deriving the mass action equations that describe the protein association in a micellar detergent phase [12]. According to these equations, a micellar detergent concentration series can be used to test for ideal-dilute solution behavior in the micellar phase. When ideal-dilute solution behavior is observed, the calculation of the standard state free energy change is a straightforward matter.

Here we carry out a series of experiments on the glycophorin A transmembrane dimer in micellar C14 betaine. The glycophorin A transmembrane helix is expressed in the form of a fusion protein with *Staphylococcal* nuclease, and this SNGpA99 molecule has been extensively characterized as a model system for transmembrane helix–helix interactions [3,7,15,16]. We have previously measured the standard state free energy change for this glycophorin A transmembrane dimer in micellar C_8E_5 [12]. In this study we test whether or not ideal-dilute solution behavior holds in micellar C14 betaine, a micellar environment that has recently been used in a number of studies on helix–helix interactions. In addition, we analyze a glycophorin construct containing two additional C-terminal lysine residues, termed SNGpA101. The C-terminal lysine residues are present on the peptide whose NMR solution structure has been solved, and our results show that the addition of the two C-terminal lysines has an insignificant effect on the free energy of helix–helix association. Finally, compared to our previous energetics studies, we show that the glycophorin A transmembrane dimerization in micellar C14 betaine is less favorable than that in micellar C_8E_5.

2. Materials and methods

2.1. Sample preparation

SNGpA99 is a chimeric protein composed of *Staphylococcal* nuclease followed by the transmembrane domain of human glycophorin A (GpA) terminating at position 99. The SNGpA101 construct is also analyzed in this study, and it differs slightly from the SNGpA99 chimera used in previous studies by the addition of two C-terminal lysine residues. Since the solution NMR structure of the transmembrane dimer contains these additional C-terminal lysines, we carried out experiments to determine their contribution to the self-association energetics. The *Staphylococcal* nuclease–glycophorin A transmembrane domain fusion protein has been used extensively by our group and by Engelman and coworkers to explore the sequence dependence of GpA transmembrane dimerization [3,7,15,16]. Both the SNGpA99 and SNGpA101 fusion proteins have previously been described in detail and were purified using published protocols [3]. Immediately before sedimentation equilibrium analysis, samples were exchanged by ion-exchange chromatography into buffer containing C14 betaine. The final detergent concentration for each experiment is indicated.

2.2. Sedimentation equilibrium analytical ultracentrifugation

Sedimentation equilibrium experiments were performed at 25 °C using six-sector cells and a Beckman XL-A analytical ultracentrifuge. The samples were centrifuged for lengths of time sufficient to achieve equilibrium. Data obtained from absorbance at 230 nm were analyzed by nonlinear least-squares curve fitting of radial concentration

profiles using the Windows version of NONLIN [17].

The simplest model that describes the data contained monomer, dimer and tetramer terms according to the following equation:

$$c_i = c_{\mathrm{ref}} \exp\left[\sigma(\xi_i - \xi_{\mathrm{ref}})\right] + c_{\mathrm{ref}}^2 K_{1,2} \exp\left[2\sigma(\xi_i - \xi_{\mathrm{ref}})\right] + c_{\mathrm{Tet}} \exp\left[4\sigma(\xi_i - \xi_{\mathrm{ref}})\right] + \mathrm{offset} \quad (1)$$

where c_i is the total absorbance at a radial position, r_i; c_{ref} and c_{Tet} are the monomer and tetramer absorbance values, respectively, at a reference position, r_{ref}; σ is the reduced molecular weight defined by Yphantis [18] and equals $M(1-\bar{\upsilon}_{\mathrm{Pr}}\rho)\omega^2/RT$; M is the monomer molecular weight; $\bar{\upsilon}_{\mathrm{Pr}}$ is the protein partial specific volume; ρ is the solvent density; ω is the angular velocity (radians s^{-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 and the 'offset' is a baseline term for non-sedimenting material.

The vast majority of the transmembrane protein was found to be participating in a dynamic and reversible monomer–dimer equilibrium. As in past studies [3], we also detected the presence of small amounts of tetrameric protein, and the fraction of tetramer observed in all experiments was equal to or less than 10%. Because the tetramer is not well described by a global equilibrium constant, we conclude that it represents a small amount of aggregated protein. While the inclusion of the tetramer term results in an improvement in the fitting statistics, its presence does not significantly influence the fitted value we obtain for the monomer–dimer equilibrium constant. The reliability of the global $K_{1,2}$ value in the presence of small amounts of irreversible tetramer formation is indicated by the fact that global analysis of nine data sets (three loading concentrations at three rotor speeds) is highly reproducible and generates random residuals and statistically significant parameter ($K_{1,2}$) estimates.

2.3. Interpretation of buoyant molecular weight

Since the sedimenting particle contains protein as well as associated detergent molecules, the buoyant molecular weight contains contributions from both components according to the following relationship [19–21]:

$$M(1-\phi'\rho) = M_{\mathrm{P}}(1-\bar{\upsilon}_{\mathrm{p}}\rho) + nM_{\mathrm{det}}(1-\bar{\upsilon}_{\mathrm{det}}\rho) \quad (2)$$

where ϕ' equals the partial specific volume of the protein–micellar detergent complex, $\bar{\upsilon}_{\mathrm{p}}$ equals the partial specific volume of the protein (ml g^{-1}), ρ equals the solvent density (g ml^{-1}), n is the number of detergent molecules bound to the protein and M_{det} and $\bar{\upsilon}_{\mathrm{det}}$ are the molecular weight and partial specific volume of micellar detergent associated with the protein. For measurements of protein association in the micellar phase, it is desirable to carry out experiments under conditions where effective density of the micellar detergent, $1/\bar{\upsilon}_{\mathrm{det}}$, is equal to the solvent density, ρ, since the contribution of detergent to the buoyant molar mass then becomes negligible. To a high degree of accuracy, the following relationship is then true:

$$M(1-\phi'\rho) = M_{\mathrm{P}}(1-\bar{\upsilon}_{\mathrm{p}}\rho) \quad (3)$$

For thermodynamic measurements it is preferable to match the micelle density with heavy water as opposed to additives such as sucrose, since matching with heavy water should minimally perturb the water activity [19–21]. In addition, this strategy avoids approximations for changes in the protein partial specific volume that must be made when the buoyant density of the hydrated detergent micelle is matched with other compounds [22]. Under density matching conditions with heavy water, it is straightforward to calculate the protein monomeric molecular masses and partial specific volume using Sednterp [23].

C14 betaine was purchased from Sigma-Aldrich. The effective density of the C14 betaine micelles was measured by an H_2O/D_2O series in the presence of all other buffering components (20 mM sodium phosphate, pH 7.0, 220 mM NaCl). In agreement with previous work [24], the match-

ing D_2O content was found to be 13% (v/v) at 25 °C.

The seven detergent concentrations employed were 4, 5.6, 7.8, 11, 15, 21 and 30 mM C14 betaine. The concentration of detergent in micellar phase was calculated by subtracting the critical micelle concentration (0.4 mM, according to Calbiochem) from each of the total detergent numbers above. For each global fit (at each detergent concentration), nine equilibrium data sets were collected. These consisted of three different initial protein concentrations analyzed at three significantly different speeds (20 000, 24 500, 30 000) (such that the speed factor ratios were at least 1.0, 1.5 and 2.25).

3. Results

3.1. The protein-C14 betaine micellar detergent phase behaves like an ideal-dilute solution

We have previously described the mass action equations that describe the protein association in a micellar detergent phase [12]. This formalism incorporates the approximations that the micellar detergent forms a distinct phase of matter that is dispersed in the aqueous phase [25]. Conceptually, this thermodynamic assumption is equivalent to assuming the presence of one large micelle within which the transmembrane protein equilibrates [25]. Using these equations, we showed that the $\partial \ln K_{App}/\partial \ln[\text{micellar detergent}]_w$ should be equal to negative unity if the protein association reaction is behaving ideally in the micellar detergent phase [12]. We have shown that this behavior is true for the glycophorin A dimerization reaction in micellar solutions of C_8E_5 [12].

Other groups have begun to study the transmembrane helix–helix oligomerization of designed peptides in micellar solutions of C14 betaine [6,8,24]. To compare the energetics of glycophorin A dimerization with these studies, we carried out a series of experiments to determine whether or not ideal solution behavior is observed in the zwitterionic detergent C14 betaine. Fig. 1 shows the results of glycophorin A dimerization measured by two independent detergent concentration series. These data demonstrate that the slope of the $\ln K_{App}$ dependence on $\ln[\text{micellar C14 betaine}]_W$ is indeed negative unity for glycophorin A dimerization in this hydrophobic environment.

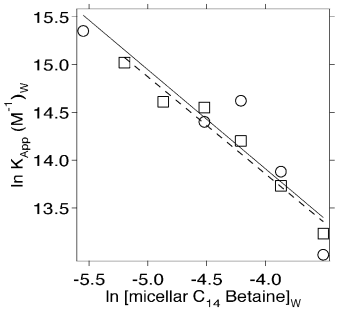

Fig. 1. The dependence of K_{App} on $\ln[\text{micellar } C_{14} \text{ betaine}]_W$ for the SNGpA101 construct. Apparent association constants are plotted as a function of micellar C14 betaine concentration. The circles and squares indicate two independent concentration series. The slopes for linear fits to these data are -1.04, $R=0.93$ (solid line through circles) and -1.02, $R=0.98$ (broken line through squares). The detergent concentrations used for the series are 5.6, 7.8, 11, 15, 21 and 30 mM for the series shown in squares and 4, 11, 15, 21 and 30 mM for the series shown in circles.

3.2. The standard state free energy change for the glycophorin A dimerization reaction in C14 betaine

Since the slope in Fig. 1 is negative unity, one can extrapolate to find the value of the mole fraction standard state free energy change as shown in Fig. 2, which uses the following equation:

$$\Delta G_{App} = \Delta G^{\circ}_{x} + RT \ln[\text{micellar Det}]_W \quad (4)$$

where ΔG_{App} equals the apparent free energy change calculated from the experimentally obtained K_{App}, ΔG°_{x} is found as the intercept for the line in Fig. 2 and equals the standard state free energy change (on the mole fraction scale) and $[\text{micellar Det}]_W$ is the concentration of the micellar detergent expressed on the aqueous molar scale. The results for two independent concentration series show that the mole fraction standard state free energy change for SNGpA101 glycophorin A dimerization in C14 betaine micelles equals -5.8 kcal mol^{-1} at 25 °C. This value is within error of

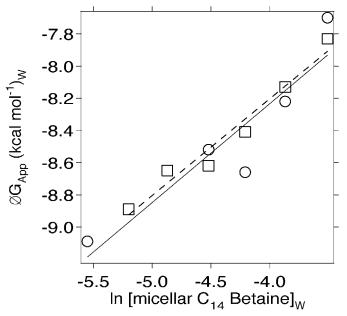

Fig. 2. Determination of standard state free energy change in C14 betaine. The circles and squares indicate two independent concentration series. The intercepts of linear fits to each of the series reveal standard state free energy changes of -5.82 and -5.78 kcal mol^{-1}. A similar analysis was carried out for the SNGpA99 construct. From five independent concentration series, the standard state free energy change for association was found to be -5.7 (± 0.3) kcal mol^{-1} (data not shown).

the value we find for the SNGpA99 glycophorin construct, which equals -5.7 (± 0.3) kcal mol^{-1} ($N=5$ independent concentration series).

4. Discussion

One of the key questions in understanding how helical membrane proteins fold is determining how the balance of forces specifies and stabilizes transmembrane α-helical bundles. Insight into the role played by the hydrophobic environment in this process may be gained by observing how changes in the environment influence a particular equilibrium ensemble of helical oligomers. The availability of a clear method for calculating a standard state free energy change simplifies comparisons between different hydrophobic environments.

We have previously shown that the standard state free energy change for the SNGpA99 glycophorin A dimerization is -7.0 kcal mol^{-1} in C_8E_5 micelles. Here, we have used a concentration series to experimentally show that the glycophorin A transmembrane helix dimerization reaction behaves ideally in micellar solutions of C14 betaine. Because we observed the ideal behavior, we have been able to determine that the $\Delta G^{\circ}_{x} = -5.7$ kcal mol^{-1} for this construct in micellar C14 betaine at 25 °C. We additionally show that the two additional C-terminal lysine residues, which are present in the glycophorin A NMR structure, have no significant effect on the energetics of self-association.

A comparison of the standard state free energy changes should inform on the energetic perturbation due to changing the hydrophobic environment. The mole fraction distributions of the glycophorin A transmembrane dimer in micellar C_8E_5 and C14 betaine are shown in Fig. 3. The results show that glycophorin A dimerization is more favorable in C_8E_5 micelles than that in C14 betaine micelles. In a sense this finding suggests that C14 betaine micelles are a better solvent for the monomeric glycophorin A transmembrane peptide than C_8E_5 micelles. Since the two micellar environments differ in many aspects of their chemistry, including chain length and head group polarity, the reasons underlying the differences in energetics may be numerous. Even though a more systematic study will be required to understand the perturbations in detail, the observation of ideal-dilute behavior in C14 betaine suggests that it is a hydrophobic solvent in which thermodynamic measurements can be accurately carried out.

A comparison of the energetics for glycophorin A association with previously published work on other sequences suggests that the glycophorin A association may be more favorable by several kilocalorie per mole when compared on a per-interface basis. DeGrado and coworkers have also used sedimentation equilibrium analytical ultracen-

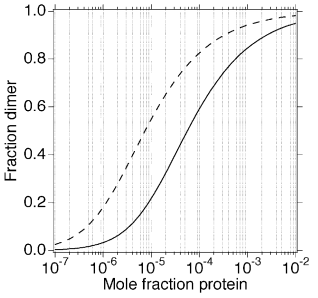

Fig. 3. Comparison of the dimerization propensities for glycophorin A in C_8E_5 vs. C14 betaine. The fraction dimer is shown as a function of mole fraction of total protein. The distribution for C_8E_5 is given by the broken line, and the distribution in C14 is given by the solid line.

trifugation to measure the free energies of association for several peptide sequences in C14 betaine micelles [6,8,24]. These sequences are a designed transmembrane version of the GCN4 peptide. The parent sequence is termed MS1 and was found to trimerize in micellar C14 betaine with a mole fraction free energy of trimerization equal to -6.8 kcal mol^{-1}. While comparisons of association energetics between oligomers of differing stoichiometries can be tricky, as a first approximation, we will assume a structural model for the MS1 peptide in which it forms a side-to-side symmetric, trimeric bundle of helices where three equivalent interfaces are created upon trimerization. This is in contrast to the single interface that is created upon glycophorin A dimerization. Using this structural model for comparison, the free energy of association per MS1 interface equals -2.3 kcal mol^{-1}. This value is significantly less than the -5.7 kcal mol^{-1} measured for the glycophorin A dimer. A sequence variant of the MS1 peptide with Val^{7}Asn mutation trimerizes more strongly (estimated $K_{\mathrm{Diss,X}} \sim 10^{-10}$). Using our structural model for analysis, this tight association suggests a free energy of association per helix–helix interface of -4.2 kcal mol^{-1}, which is -1.5 kcal mol^{-1} less favorable than that of glycophorin A transmembrane interface. Other mutants of the MS1 peptide have even less favorable free energies of association. Thus, although the introduction of a polar Asparagine residue does increase the propensity for the MS1 peptide to self-associate, the optimized van der Waals packing of the glycophorin A transmembrane dimer still specifies the more favorable association free energy when compared on a per interface basis.

In conclusion, we have shown that the glycophorin A transmembrane association in micellar C14 betaine behaves ideally. We are thus able to calculate a standard state free energy of association equal to -5.7 (± 0.3, $N=5$) kcal mol^{-1} for the SNGpA99 construct. This value is destabilized by 1.3 kcal mol^{-1} with respect to the free energy of association in C_8E_5 micelles. However, in C14 betaine micelles, formation of the glycophorin A dimeric interface may be significantly more favorable than that of currently designed peptides. Additional experiments on a variety of transmembrane sequences and in a variety of hydrophobic environments should help define chemical principles underlying these differences in association propensities. Sedimentation equilibrium studies will undoubtedly play a major role in these studies.

Acknowledgments

We gratefully acknowledge financial support from the National Institutes of Health (R01-GM57534) as well as a Career Development Award from the Department of Defense (DAMD 17-02-1-0427). We thank Walter F. Stafford III at the Boston Biomedical Research Institute for generously providing access to his Beckman XL-I instrument to carry out the C14 betaine density matching experiments, which required interference optics. This work is dedicated to the excellent group of scientists from the University of Connecticut Analytical Ultracentrifugation Center, which was headed for many years by David Yphantis.

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