J Membrane Biol (2009) 230:49–55
DOI 10.1007/s00232-009-9185-1

Hill Coefficient Analysis of Transmembrane Helix Dimerization

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Received: 27 May 2009 / Accepted: 11 June 2009 / Published online: 15 July 2009

Abstract Here, we employed the Hill equation, used broadly to characterize cooperativity in protein–ligand binding, to describe the dimerization of transmembrane (TM) helices in hydrophobic environments. The Hill analysis of wild-type fibroblast growth factor receptor 3 (FGFR3) TM domain dimerization gives a Hill coefficient of ~ 1 for lipid bilayers but only ~ 0.2 for sodium dodecyl sulfate (SDS) micelles. We propose that this finding is indicative of heterogeneity in FGFR3 TM dimer structure and stability in SDS micelles. We further speculate that (1) the Hill equation can be used as a tool to assess the existence of multiple structural states of TM dimers in different hydrophobic environments and (2) the structural heterogeneity, detectable by Hill analysis, may be the underlying reason for the broad peaks and the low resolution NMR studies of peptides in detergents.

Keywords Lipid–protein interaction · Membrane biophysics · Transmembrane helix · Thermodynamics · Dimerization

Introduction

Despite the fact that 30% of the open reading frames in organisms encode membrane proteins (Liu and Rost 2001; Wallin and von Heijne 1998), our knowledge of their folding and their structure–function relationship is limited (MacKenzie 2006; White et al. 2001; White and Wimley 1999; Popot and Engelman 2000), due to limited biophysical tools used in the studies (White et al. 1998; MacKenzie 2006). The limitations are obvious in the studies of the lateral association of transmembrane (TM) helices, the simplest yet a critically important interaction that occurs in the hydrophobic membrane environment. While methods are now available to measure the energetics of TM helix dimerization (i.e., dimer stability) in vesicles and supported lipid bilayers (Li et al. 2005, 2006; You et al. 2006) and in bacterial membranes (Duong et al. 2007; Finger et al. 2006), high-resolution TM dimer structures are often sought in detergents. However, only a few high-resolution structures have been solved so far in detergent micelles, including the structure of the glycophorin A (GpA) TM dimer in DPC (MacKenzie et al. 1997) and the structure of the ζζ TM dimer in 5:1 DPC:SDS (Call et al. 2006), due to challenges associated with broad NMR peaks and low experimental resolution.

A review article by Mathews et al. (2006) reminds us that "the constraints of the lipid bilayer are poorly imitated by the detergents used to study membrane proteins." A question therefore arises whether the detergent environment may significantly alter the association of TM helices such that the structure and interactions in detergents and in membranes are principally different (Walkenhorst et al. 2009). Do TM helices, which form structurally well-defined dimers in membranes, exist in multiple structural states in detergent micelles such that the dimer does not have a well-defined structure in detergent? Can this be the reason for the poor resolution in the NMR experiments?

Here, we address the above questions using the FGFR3 TM domain as a model dimerizing helix. Dimerization of the FGFR3 TM domain has been studied extensively in

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lipid systems of various compositions (You et al. 2006, 2007; Li et al. 2006; Merzlyakov and Hristova 2008). FGFR3 is a receptor tyrosine kinase which conducts biochemical signals across the plasma membrane via lateral dimerization. Its TM domain has a propensity to form sequence-specific dimers in bilayers, but this propensity is rather weak (Li et al. 2005). FGFR3 TM domain mutations, such as the Ala391 → Glu mutation linked to Crouzon syndrome with acanthosis nigricans, increase the stability of the dimer (Li and Hristova 2006; Li et al. 2006). The dimerization of wild-type FGFR3 TM domain and all studied mutants in bilayers is well described as an equilibrium reaction (Li et al. 2005, 2006; You et al. 2005; Merzlyakov et al. 2006a, 2006b). Thus, the FGFR3 TM domain is a good model protein for the current study because (1) it is very "well-behaved" in bilayer systems and (2) its dimerization propensity is weak, and therefore the nature of the hydrophobic environment may affect the stability and the structure of the dimer.

Here, we use the Hill equation formalism as a quantitative tool to gain new knowledge about FGFR3 TM domain dimerization in both detergents and lipid bilayers. We show that the Hill equation can be used to describe FGFR3 TM domain interactions in both hydrophobic environments. The Hill analysis of wild-type FGFR3 TM domain dimerization gives a Hill coefficient of ~ 1 for lipid bilayers but only ~ 0.2 for SDS micelles. We interpret these results as an indication for FGFR3 dimer heterogeneity in detergent micelles, and we discuss the implications of this finding. Also, we speculate that (1) the Hill equation can be used as a tool to assess the existence of multiple structural states of TM dimers in different hydrophobic environments and (2) the heterogeneity, detected using the Hill coefficient analysis presented here, may be the underlying reason for the broad peaks and the low resolution in NMR experiments in detergents.

Materials and Methods

Hill Coefficient Analysis of TM Helix Dimerization

The dimerization of TM helices is described by an association constant, K, defined as

$$K = \frac{[D]}{[M]^2} \tag{1}$$

where $[M]$ and $[D]$ are the monomer and dimer concentrations, respectively. If $[T] = 2[D] + [M]$ is the total protein concentration, the fraction of proteins in the dimeric state is

$$f = \frac{2[D]}{[T]} = \frac{2[M]}{2[M] + 1/K} \tag{2}$$

Now, we rearrange Eq. 2 and obtain

$$\frac{f}{1-f} = 2K \cdot [M] \tag{3}$$

Taking the logarithm, we write

$$\ln\left(\frac{f}{1-f}\right) = \ln(K) + \ln[2M] \tag{4}$$

Equation 4 follows directly from Eq. 1, and therefore it also describes an equilibrium between monomers and dimers.

We now examine Eq. 4 more closely by comparing it to the Hill equation. The Hill equation,

$$\ln\left(\frac{\Theta}{1-\Theta}\right) = \ln(K) + n\ln[L] \tag{5}$$

is frequently used in biochemistry to describe multivalent ligand binding to proteins. In this equation Θ is the fraction of protein binding sites filled with the ligand, K is the binding constant, $[L]$ is the concentration of ligand and n is the Hill coefficient, which describes the cooperativity of binding. If binding does not depend on the number of ligands bound to the protein, binding is considered "non-cooperative" and the Hill coefficient has a value of 1. A value greater than 1 indicates positive cooperativity (binding is enhanced by the presence of other ligands), while a value less than 1 means negative cooperativity (binding affinity decreases once a ligand is bound).

While the Hill equation was derived to describe the cooperative binding of oxygen to hemoglobin (Hill 1910), comparison between Eqs. 4 and 5 suggests that it may also be used to describe TM helix dimerization. We see that Eq. 4 is very similar to the Hill equation, with $n = 1$ and $L = 2M$, suggesting that the association of two monomers into a dimer can be considered analogous to the binding of a ligand to a protein binding site. The comparison suggests that the fraction of TM helices in the dimeric state, f, is equivalent to the fraction of protein binding sites occupied by the ligand, i.e., $f = \Theta$, and the concentration of free ligand is equivalent to twice the concentration of monomers (free "ligands"), $[L] = 2[M]$. The latter can be rationalized because two monomers are required to form a TM helix dimer, but only one ligand is needed for protein–ligand binding.

In studies of protein–ligand interactions, measured values of $\ln[\Theta/(1-\Theta)]$ as a function of $\ln(L)$ are fitted to Eq. 5 to obtain n and determine the degree of cooperativity. Can a similar fit be used to provide new insights into the TM helix dimerization process? With this question in mind,

we rewrite Eq. 4 in a more generalized way, following the well-established Hill analysis formalism:

$$\ln\left(\frac{f}{1-f}\right) = \ln(K) + n \cdot \ln[2M] \quad (6)$$

Within the Hill formalism, this equation can be fitted to experimental measurements of $\ln[f/(1-f)]$ as a function of $2M$ to determine n.

Förster Resonance Energy Transfer Measurements of Dimerization in Detergent

The Förster resonance energy transfer (FRET) efficiency between FGFR3 TM domains labeled with either fluorescein (donor) or rhodamine (acceptor) was measured as described (You et al. 2005). The peptide concentration range was 4–400 μM, and the SDS concentration was 200 mM. High label concentrations, such as the ones required in this study of weak interactions, lead to inner-filter effects (i.e., absorbance of the excitation and emission light by the sample), even when measurements are performed in a 10-μl microcuvette. To minimize this effect, a small sample volume (4 μl) was sandwiched between two quartz slides, resulting in a path length of approximately 40 μm or less. The slides were mounted on a homemade slide holder (Merzlyakov et al. 2006a). Emission and excitation scans, such as the spectra shown in Fig. 1a and b, were recorded with a Fluorolog-3 fluorometer (Jobin Yvon, Edison, NJ). The background signal from the quartz slides and the detergent was below 15,000 cps and was subtracted from the measured spectra prior to data evaluation. The FRET efficiency was calculated as shown in Fig. 2. The calculation is based on comparison of the FRET emission and excitation spectra to standard spectra of the donor and the acceptor at known concentrations (not

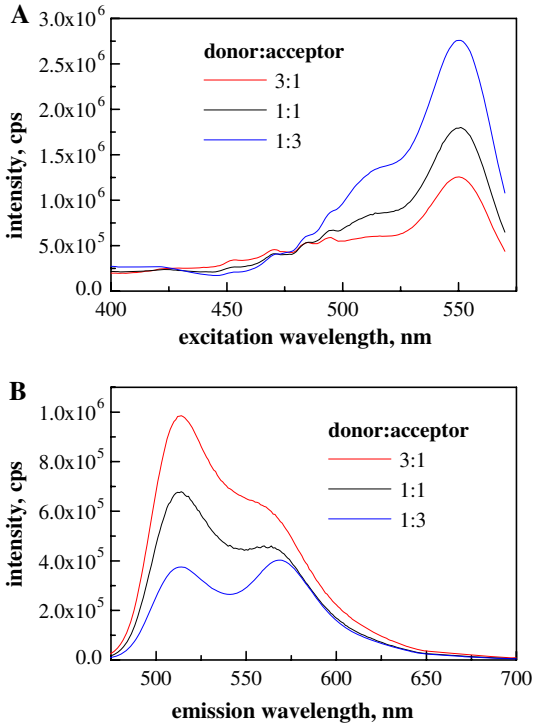

Fig. 1 Excitation (**a**) and emission (**b**) spectra, recorded for 200 μM Ala391Glu TM in 200 mM SDS at different donor to acceptor ratios

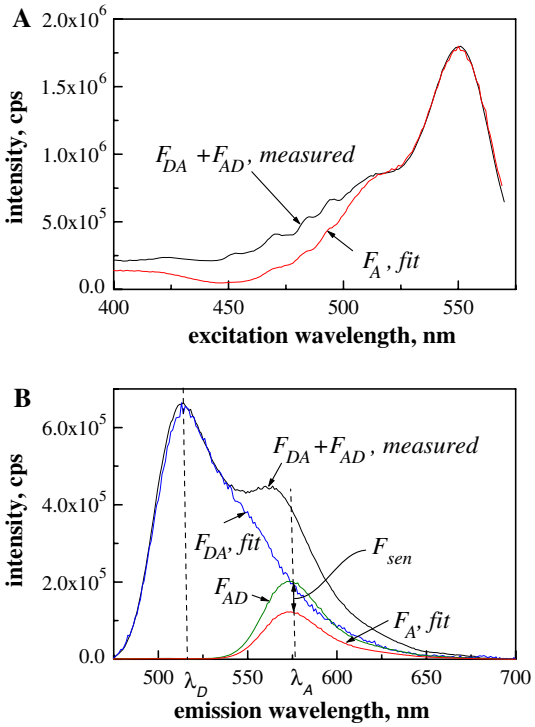

Fig. 2 FRET efficiency calculation from fluorescence spectra. The calculation is based on comparison of the FRET emission and excitation spectra to standard spectra of the donor and the acceptor at known concentrations (not shown). F_{DA} and F_D are the measured fluorescence spectra of the donor in the presence and absence of the acceptor; F_{AD} and F_A are the measured fluorescence spectra of the acceptor in the presence and in absence of the donor; λ_D are λ_A are the emission peak wavelengths of the donor and the acceptor, respectively. *Black curves* show the measured FRET excitation spectrum (**a**) and the FRET emission spectrum (**b**) of 200 μM Ala391Glu TM in 200 mM SDS (donor to acceptor ratio of 1). The background signal from the quartz slides and the detergent is below 15,000 cps and was subtracted from the measured spectra prior to data evaluation. To calculate the FRET efficiency, the measured FRET excitation spectrum is fitted to the standard acceptor excitation spectrum at wavelengths longer than 530 nm where the donor is not excited. This step gives the direct emission of the acceptor, F_A. Next, the FRET emission spectrum is split into two components, F_{DA} and F_{AD}, by fitting the standard donor emission spectrum to the measured FRET spectrum, $F_{DA} + F_{AD}$, at wavelengths shorter than 520 nm where the acceptor is not emitting. Finally, the sensitized fluorescence of the acceptor, F_{sen}, is determined as $F_{sen} = F_{AD} - F_A$, and the FRET efficiency is calculated as $E \cong \frac{F_{sen}(\lambda_A)}{F_{DA}(\lambda_D)+F_{sen}(\lambda_A)}$ (see Merzlyakov et al. 2007). The donor to acceptor ratios and the acceptor fraction χ_a are determined by comparing the amplitudes of F_D and F_A to the standard spectra (see Merzlyakov et al. 2007, for details)

shown). To calculate the FRET efficiency, the measured FRET excitation spectrum is fitted to the standard acceptor excitation spectrum at wavelengths longer than 530 nm where the donor is not excited. As discussed by Merzlyakov et al. (2007) and Merzlyakov and Hristova (2008), this step gives the direct emission of the acceptor in the absence of the donor, F_A. Next, the FRET emission spectrum is split into two components, the spectra of the donor and the acceptor in the presence of their FRET partners, F_{DA} and F_{AD}. This was accomplished by fitting the standard donor emission spectrum to the measured FRET spectrum, $F_{DA} + F_{AD}$, at wavelengths shorter than 520 nm where the acceptor is not emitting. Finally, the sensitized fluorescence of the acceptor, F_{sen}, is determined as $F_{sen} = F_{AD} - F_A$, and the FRET efficiency is calculated as $E \cong \frac{F_{sen}(\lambda_A)}{F_{DA}(\lambda_D)+F_{sen}(\lambda_A)}$ (see Merzlyakov et al. 2007, for details). The donor-to-acceptor ratios and the acceptor fraction, χ_a, are determined by comparing the amplitudes of F_D and F_A to the standard spectra (Merzlyakov et al. 2007).

The measured FRET efficiency was corrected for FRET that arises due to random colocalization of donors and acceptors (proximity effects) (Li et al. 2006; You et al. 2005; Posokhov et al. 2008). An estimate of this proximity contribution was obtained by measuring FRET between fluorescein- and rhodamine-labeled lipids in SDS micelles (data not shown). Since there are no specific interactions between lipids, the observed FRET between labeled lipids is only due to random colocalization of the two dyes. Under all conditions studied, this contribution did not exceed $\sim$15% of the measured FRET.

Results

Analysis of Dimerization in Bilayers

We first verify that Eq. 4 holds for both wild-type FGFR3 TM domain and the Ala391Glu mutant, using previous FRET measurements in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) liposomes. FRET measurements give the dimer and monomer concentrations, $[D]$ and $[M]$, as a function of the total concentration $[T]$. In Fig. 3 we replot published data (Li et al. 2006), plotting measured values of $\ln[f/(1-f)]$ as a function of $\ln[2M]$ (shown as symbols in Fig. 3a for the wild-type and in Fig. 3b for the Ala391Glu mutant). Since the equilibrium constants, K, had been measured as well, we also plot the function $\ln(K) + \ln[2M]$ as a function of $\ln[2M]$ (solid lines in Fig. 3a, b). Figure 3 shows that, despite scatter, the data are generally consistent with the theoretical curves given by Eq. 4.

Alternatively, we can fit the data (shown with symbols) in Fig. 3 to the generalized Eq. 6, with n being unknown. The fits are shown with the dotted lines. The calculated Hill coefficients are $n = 1.1 \pm 0.2$ for the wild-type (Fig. 3a) and $n = 0.8 \pm 0.4$ for the mutant (Fig. 3b).

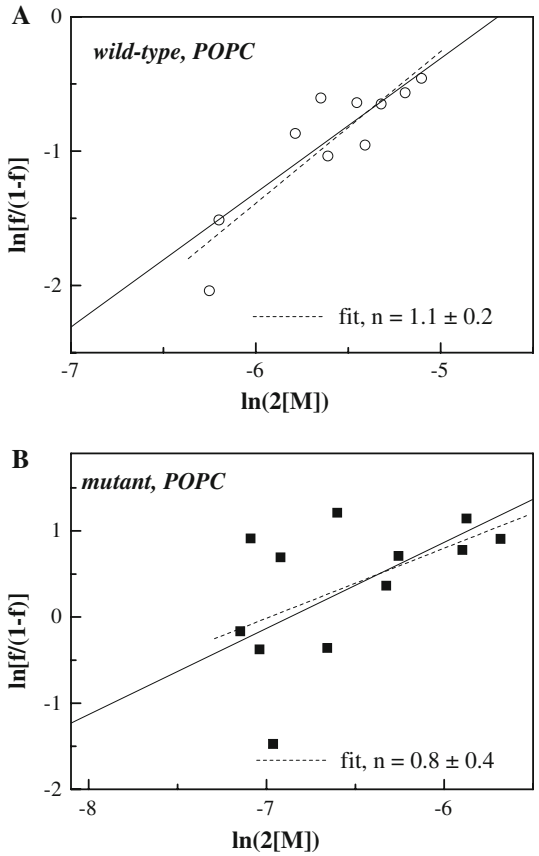

Fig. 3 Hill plots for POPC: Natural logarithm of $f/(1-f)$ vs. the logarithm of $2[M]$, where f is the fraction of peptides in the dimeric state, for the wild-type FGFR3 TM domain (**a**) and for the Ala391Glu mutant (**b**). Values for $[M]$, $[D]$ and $[T]$ are from Li et al. (2006). Experimental data were fitted to the generalized Eq. 6, with n being the unknown. The fit is shown with the *dotted line* and it gives $n = 1.1 \pm 0.2$ for the wild-type (**a**) and $n = 0.8 \pm 0.4$ for the mutant (**b**). The slope of the *solid lines* equals 1 ($n = 1.0$)

Thus, the presented analysis shows that the Hill equation, with L substituted by $2M$, describes the dimerization of FGFR3 TM domain in POPC vesicles. Furthermore, the Hill coefficients obtained for both wild-type FGFR3 TM domain and the Ala391Glu mutant are close to 1.

Analysis of Dimerization in Detergent

Next, the dimerization of wild-type FGFR3 TM domain and that of the Ala391Glu mutant were studied in SDS following the protocol described in "Materials and Methods." The FRET efficiency was calculated as shown in Fig. 2. The measured FRET efficiency was corrected for FRET that arises due to random colocalization of donors and acceptors (proximity effects, see "Materials and Methods") (Li et al. 2006). As discussed previously (Adair and Engelman 1994; Merzlyakov et al. 2007), the corrected

FRET efficiency, E, is proportional to $1 - (1 - \chi_a)^{m-1}$, where χ_a is the acceptor fraction and m is the oligomer size ($m = 2$ for a dimer). Therefore, measurements of E as a function of χ_a at a constant peptide concentration give the size of the oligomer (Adair and Engelman 1994; Li et al. 1999). In particular, a linear dependence of E on χ_a is indicative of dimer formation and the existence of monomers and dimers only. Such linear dependence is shown in Fig. 4a for the wild-type and in Fig. 4b for the mutant, demonstrating that both peptides form dimers in detergent ($m = 2$).

To create the Hill plots, we calculated the fraction of peptides in the dimeric state, f, as $f = E/\chi_a$, while keeping the donor-to-acceptor ratio constant at 1:1 and varying the total peptide concentration [T]. The total peptide concentration [T] was known as aliquoted, and the monomer concentration was calculated as $[M] = [T](1 - f)$. Figure 5 shows the Hill plots for the wild-type FGFR3 TM domain and the Ala391Glu mutant in SDS. Experimental values (shown with symbols) were fitted to Eq. 6. The solid lines are the fits, with Hill coefficient values of $n = 0.16 \pm 0.04$ for the wild type and $n = 0.8 \pm 0.4$ for the Ala391Glu mutant.

Thus, while the Hill coefficient for the Ala391Glu mutant is ~ 1 in both lipid vesicles and detergents, the value for the wild type in detergent is ~ 0.2. In the formalism of the Hill equation, the latter corresponds to anticooperative binding.

Discussion

Previously, we showed that the FGFR3 TM domain forms dimers in POPC bilayers (Li et al. 2005, 2006). Now, we show that this dimerization process can be described by the Hill equation, with Hill coefficient equal to 1. We also find that FGFR3 forms dimers in SDS (see Fig. 4). The Hill coefficient for the wild-type FGFR3 TM domain, however, is only 0.16 ± 0.04 in SDS (Fig. 5). We propose that this finding is indicative of heterogeneity in the interactions between the helices in the wild-type FGFR3 dimer in SDS.

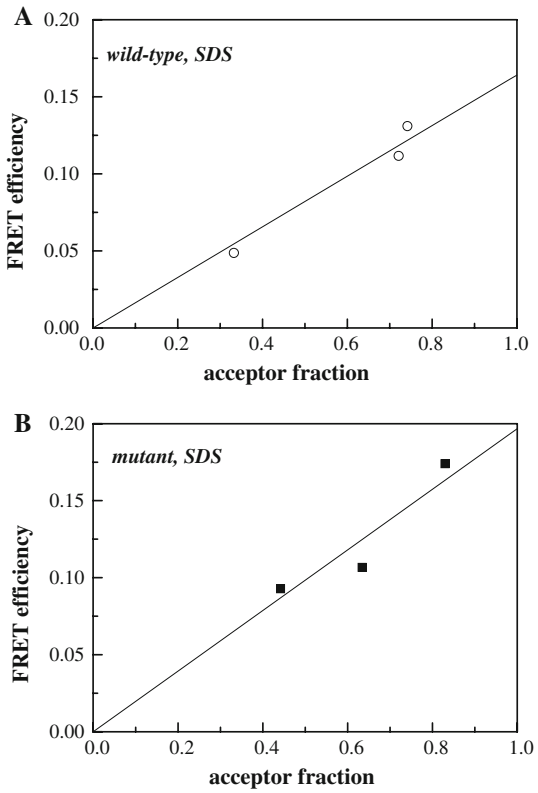

Fig. 4 FRET efficiency as a function of acceptor fraction, χ_a, for two different samples: (**a**) 4 μM wild-type FGFR3 TM domain in 200 mM SDS and (**b**) 200 μM Ala391Glu in 200 mM SDS. The acceptor fraction was determined as shown in Fig. 2. The linear dependence of the FRET efficiency on χ_a is indicative of dimer formation. Such linear dependence was observed for all peptide concentrations studied: 4, 40, 200 and 400 μM

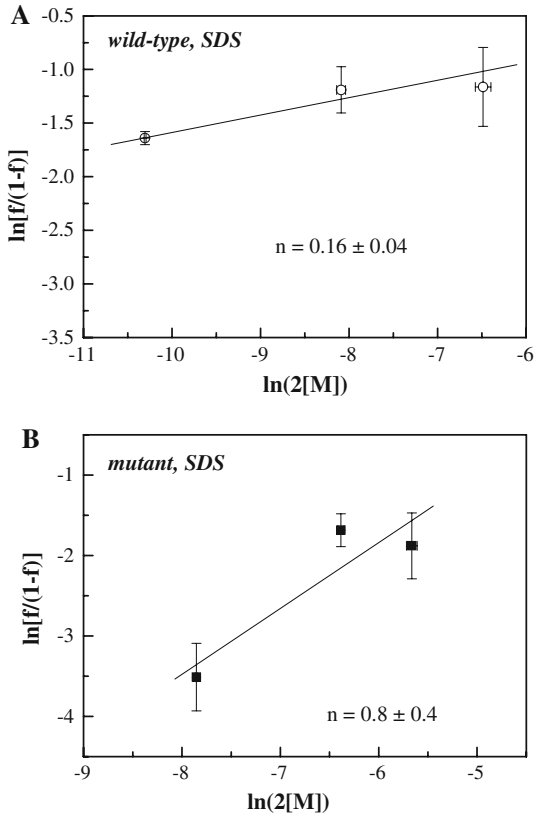

Fig. 5 Hill plots for SDS: Natural logarithm of $f/(1 - f)$ vs. logarithm of 2[M] for the wild-type FGFR3 TM domain (4, 40 and 200 μM) (**a**) and for the Ala391Glu mutant (40, 200 and 400 μM) (**b**), both in 200 mM SDS. *Solid lines* are given by Eq. 6; the Hill coefficient, n, is determined by fitting the solid lines to the experimental data. Error bars shown are the standard deviations calculated from three different experiments at a given concentration

We further propose that the observed heterogeneity in dimerization thermodynamics is associated with heterogeneity in dimer structure. This concept can be rationalized within the general concept of anticooperative binding: If multiple dimer structures exist, then the formation of a dimer interface between two TM helices will exclude all other possible interactions between them. Within this framework, the measured Hill coefficient of ~ 1 in POPC bilayers is indicative of a single dimeric state with well-defined structure and stability. A single dimeric structure is also observed for the Ala391Glu mutant in both lipids and detergents, most likely stabilized by Glu391-mediated hydrogen bonds. Note that n is the number of binding sites and, thus, the maximum value of n is 1 in the context of dimerization.

Despite the fact that wild-type FGFR3 TM domain forms "well-behaved" dimers in lipid bilayers, its dimers in SDS are heterogeneous. While the exact nature of this heterogeneity is unknown, it can be expected that a family of different dimer structures exists and that these structures are associated with different dimerization propensities. Furthermore, SDS micelles may contain both parallel and antiparallel dimers, exhibiting different FRET efficiencies. It is also possible that the micelle population is heterogeneous (Walkenhorst et al. 2009) such that the peptides encounter different environments, and this in turn leads to different structures and stabilities. We propose that such structural heterogeneity in detergents is not limited to the FGFR3 TM domain in SDS but is a general feature of weakly bonded TM dimers in detergents. We further propose that this heterogeneity may be the underlying reason for the broad peaks and the low resolution in NMR detergent experiments. Thus, the presented Hill analysis may provide a means to evaluate the likelihood of success in dimer structure determination in detergent using NMR. We note that the Hill analysis of glycophorin A dimerization in DPC (data of Fisher et al. 1999) gives a Hill coefficient of $n = 1.03 \pm 0.04$, consistent with this view (see Fig. 6).

In summary, detergent micelles may be unsuitable for structural and thermodynamic characterization of some noncovalently bonded TM dimers (particularly weak dimers), and the presented method is a tool to evaluate the utility of a given detergent system in the characterization of a particular TM helix dimer. The structure and stability of weak TM dimers in detergents may be different from the structure and stability in membranes, and therefore, experiments in detergents may not report on the native interactions in the cellular membrane. It should be kept in mind that the presented measurements are carried out in SDS, a detergent that is known to denature membrane proteins; and thus, the discussions about the suitability of different detergent systems in studies of membrane proteins should continue. Finally, it should be noted that lipid bicelles have been used to solve the structure of TM dimers in the lipid environment (Bocharov et al. 2007, 2008a, 2008b), thus demonstrating that lipid systems are a viable alternative to detergent solutions in the quest for high-resolution TM dimer structures.

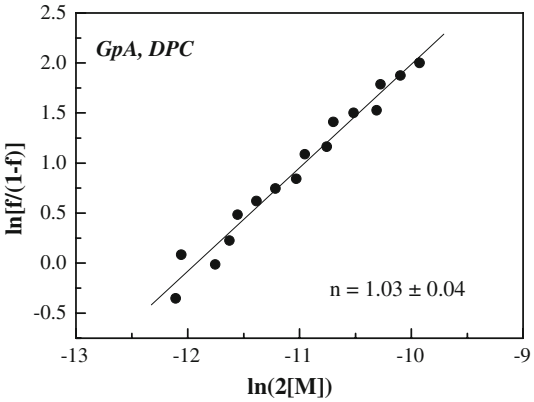

Fig. 6 Hill plots for glycophorin A (GpA) in DPC, replotted data from Fisher et al. (1999). The structure of the GpA dimer in DPC has been solved by NMR (MacKenzie et al. 1997), and the analysis of previously published energetics measurements (Fisher et al. 1999) gives a Hill coefficient of 1

Acknowledgments This work was supported by NIH grant GM068619 and Research Scholar Grant RSG-04-201-01 from the American Cancer Society (to K. H.).

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