Published in final edited form as:

J Mol Biol. 2006 April 21; 358(1): . doi:10.1016/j.jmb.2006.01.086.

Transmembrane helix heterodimerization in lipid bilayers: probing the energetics behind autosomal dominant growth disorders

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SUMMARY

Here we show that the energetics of TM helix heterodimer formation can be characterized in liposomes using FRET. We present the theory and the protocol for measuring the free energy of heterodimerization, and the total (hetero and homo-dimeric) dimer fraction. We use the presented methodology to determine the propensity for heterodimer formation between wild-type FGFR3 TM domain and the Ala391Glu mutant, linked to Crouzon syndrome with acanthosis nigricans.

Keywords

heterodimer; FRET; Receptor tyrosine kinase; fibroblast growth factor receptor 3; transmembrane domain

Introduction

Studies of TM helix homodimerization have shed light on the thermodynamic and structural determinants of the self-association process in hydrophobic environments¹⁻⁴. Investigations of TM helix heterodimerization, however, are scarce, and the physical-chemical characteristics of the process are just beginning to emerge. Heterodimerization has been studied so far using the genetic GALLEX assay⁵, in which the extent of heterodimerization in the *E. coli* inner membrane is reported by a decrease in β -galactosidase synthesis. The need remains, however, to develop alternative methods that provide precise quantitative thermodynamic information about the heterodimerization process.

Heterodimerization of TM helices is in the very heart of the membrane protein folding problem. TM helices with different amino acid sequences interact laterally to give rise to membrane proteins with complex folds, such as ion channels and G-protein coupled receptors, performing intricate biochemical tasks. Our understanding of membrane protein folding lags behind our knowledge of soluble protein folding principles, partly due to lack of adequate experimental methods^{6,7}. A method to measure the free energy of heterodimerization, therefore, would be an important tool in the studies of membrane protein folding, and the structure-function relation for membrane proteins.

The physical characterization of RTK TM homodimers has provided new insights into RTK signaling^{4,8-11}. However, the biophysics behind RTK TM heterodimer formation is largely unknown, despite the fact that RTK heterodimers do form. Furthermore, single amino acid RTK mutations that are known to cause pathological phenotypes are often dominant, and the

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cells of the affected heterozygotes express both wild-type and mutant proteins. An example of an autosomal dominant mutation is the Ala391Glu mutation in the TM domain of FGFR3, an RTK that is crucial for skeletal development^{12–14}. Fibroblast growth factor receptor 3 (FGFR3), which consists of three extracellular glycosylated Ig-like domains, a transmembrane (TM) domain, and a cytoplasmic catalytic domain, conducts biochemical signals via lateral dimerization in the plasma membrane. The TM Ala391Glu mutation causes Crouzon syndrome, characterized by premature ossification of the skull, and acanthosis nigricans, a skin disorder¹⁵. It has also been identified as a somatic mutation in bladder cancers¹⁶.

The homodimerization free energies have been measured for both wild-type and mutant (Ala391Glu) FGFR3 TM domains. The change in free energy of homodimerization due to the Ala391Glu mutation was determined as -1.3 kcal/mole¹⁰. This seemingly small value can, under certain conditions, lead to a several-fold increase in dimer fraction and could affect signaling in a profound way¹⁰. While Crouzon syndrome with acanthosis nigricans is an autosomal dominant disorder, quantitative characterization of heterodimer formation between the wild-type FGFR3 TM domain and the pathogenic Ala391Glu mutant has not been carried out. Here we discuss the thermodynamics behind heterodimer formation, and we outline a method for calculating the free energy of dimerization using FRET. We then determine the free energy for the wild-type/mutant FGFR3 TM heterodimer formation.

Theory and protocol

In a lipid bilayer with two different TM helices, X and Y, three different types of dimers, XX, YY and XY, will form (see figure 1A). The monomer-dimer equilibrium is described by three equilibrium constants, K_X , K_Y and K_{XY} :

$$K_X = \frac{[XX]}{[X]^2}, K_Y = \frac{[YY]}{[Y]^2}, K_{XY} = \frac{[XY]}{[X] \cdot [Y]} \quad (2)$$

where $[XX]$, $[YY]$, $[XY]$ are the concentrations of the XX homodimers, YY homodimers and XY heterodimers, respectively; $[X]$ and $[Y]$ are the concentrations of the X and Y monomers, respectively. All concentrations are in dimensionless units, mole of peptide per mole of lipid (i.e. peptide-to-lipid ratio)¹⁷. The homodimer equilibrium constants K_X and K_Y can be measured independently for helices X and Y as previously described^{8,10,17}.

The total concentrations of the X and Y helices, $[T_X]$ and $[T_Y]$, are known as aliquoted and equal to:

$$[T_X] = [X] + 2[XX] + [XY] \text{ or } [T_X] = [X] + 2K_X \cdot [X]^2 + [XY] \quad (3)$$

$$[T_Y] = [Y] + 2[YY] + [XY] \text{ or } [T_Y] = [Y] + 2K_Y \cdot [Y]^2 + [XY] \quad (4)$$

Let X be labeled with a FRET donor, and Y be labeled with the appropriate acceptor. The experimentally measured FRET efficiency, E_{exp} , can be calculated from the decrease in donor fluorescence (donor quenching) according to¹⁸:

$$E_{\text{exp}} = 1 - F_{DA}/F_D, \quad (5)$$

where F_{DA} and F_D are the donor fluorescence intensities in the presence and absence of the acceptor. As described previously¹⁷, the measured FRET efficiency has two contributions: (1) a contribution from sequence-specific dimerization, E_{dimer} and (2) a contribution from random colocalization of donors and acceptors (proximity effects), $E_{\text{proximity}}$:

$$E_{\text{exp}} = E_{\text{dimer}} + E_{\text{proximity}} \quad (6)$$

For TM dimers, the FRET efficiency due to sequence specific dimerization is given by^{8,17}:

$$E_{\text{dimer}} = \frac{[da]}{[T_d]}, \quad (7)$$

where $[T_d]$ is the total concentration of donors and $[da]$ is the concentration of donor-acceptor dimers. $[da]$ would be equal to $[XY]$, and $[T_d]$ would be equal to $[T_X]$ if the labeling yield is 100% (i.e. the ratio of peptides to conjugated fluorescent dyes is 1). As previously discussed¹⁷, 100% labeling is generally hard to achieve for the very hydrophobic TM domains, and therefore labeling yields need be measured and accounted for. If the labeling yields, f_D and f_A for the donor and the acceptor, are lower than 100%, then:

$$[T_d] = f_D \cdot [T_X], 0 < f_D < 1 \quad [T_a] = f_A \cdot [T_Y], 0 < f_A < 1. \quad (8)$$

Assuming that labeling does not affect the dimerization energetics, the concentration of donor-acceptor dimers relates to the total concentration of heterodimers XY as follows:

$$[da] = [XY] \cdot f_D \cdot f_A \quad (9)$$

From Eqs. (7), (8) and (9), we obtain:

$$E_{\text{dimer}} = \frac{[XY] \cdot f_D \cdot f_A}{f_D \cdot [T_X]} = \frac{[XY]}{[T_X]} f_A, \quad (10)$$

i.e. $[XY]$ can be directly measured in the FRET experiment because $[T_X]$ and f_A are known. Equations (3), (4) and (10) can be solved to determine the three unknowns: $[X]$, $[Y]$ and $[XY]$.

The solution is:

$$[XY] = E_{\text{dimer}} \cdot [T_X] / f_A \quad (11)$$

$$[X] = \frac{\sqrt{1 + 8K_X \cdot [T_X](1 - E_{\text{dimer}}/f_A)} - 1}{4K_X} \quad (12)$$

$$[Y] = \frac{\sqrt{1 + 8K_Y([T_Y] - E_{\text{dimer}} \cdot [T_X]/f_A)} - 1}{4K_Y} \quad (13)$$

The heterodimerization equilibrium constant K_{XY} is calculated as:

$$K_{XY} = \frac{E_{dimer} \cdot [T_X]}{[X] \cdot [Y] \cdot f_A}, \quad (14)$$

where $[X]$ and $[Y]$ are given by Eqs. (12) and (13), respectively. Then, the free energy of heterodimerization can be calculated as:

$$\Delta G_{XY} = -RT \ln K_{XY}. \quad (15)$$

Finally, it should be noted that the presented method allows us to directly determine if heterodimers form or not. If heterodimers do not form, the measured FRET will be due to proximity effects only. Furthermore, similar calculations can be used to determine the dimer fraction and the free energy of heterodimerization even if the ratio between donors and acceptors is not 1:1.

Monomer-dimer equilibrium and total dimer fractions

In a lipid bilayer with two different TM helices, X and Y, the total concentration of dimers is given as $[D] = [XX] + [YY] + [XY]$, whereas the total concentration of monomers is $[M] = [X] + [Y]$. The equilibrium constant K_{eq} describing the over-all dimerization process is defined as:

$$3X + 3Y \xrightleftharpoons{K_{eq}} XX + XY + YY, \quad K_{eq} = \frac{[XX] \cdot [XY] \cdot [YY]}{[X]^3 [Y]^3} = K_X \cdot K_{XY} \cdot K_Y \quad (16)$$

Note that K_{eq} is not defined via the total dimer concentration $[D]$, and is different from the ratio $[D]/[M]^2$, i.e.:

$$K_{eq} \neq \frac{[D]}{[M]^2} = \frac{[XX] + [YY] + [XY]}{([X] + [Y])^2} = \frac{K_X \cdot [X]^2 + K_Y \cdot [Y]^2 + K_{XY} \cdot [X] \cdot [Y]}{([X] + [Y])^2}. \quad (17)$$

Therefore, the total dimer fraction $[D]/[T]$, where $[D] = [XX] + [YY] + [XY]$ and $[T] = [T_X] + [T_Y]$, follows an equilibrium curve that is different from the homodimer curve. This is illustrated in Figure 1B, which shows the shape of the equilibrium curve (i.e. the total dimer fraction $[D]/[T]$ as a function of the total peptide concentration $[T]$) for homodimers and heterodimers. The solid line is the dimer fraction for a single dimerizing helix. The dashed and the dotted lines describe the total (homo- plus hetero-) dimer fraction when both X and Y are present, for two different values of $G = G_Y - G_X$ (see below and figure caption). To produce the dotted and the dashed line, we have assumed that the ratio of total concentrations $[T_X]/[T_Y] = 1$, and that the free energy of heterodimer formation is the average of G_Y and G_X i.e., $G_{XY} = (G_Y + G_X)/2$, where $G_Y = -RT \ln K_Y$ and $G_X = -RT \ln K_X$. The shape of the heterodimer curve (dashed or dotted) depends strongly on the relative values of G_Y and G_X . When G_Y is similar to G_X ($G = G_Y - G_X \sim -1$ kcal/mole for the dashed line) the shape of the equilibrium curve for the heterodimer is similar to the homodimer curve. When the difference between G_Y and G_X is large due to very different homodimerization propensities of helices X and Y, the shape of the equilibrium curve is dramatically different (dotted line, calculated for $G = G_Y - G_X = -5$ kcal/mole). Figure 1B shows that the shape of the equilibrium curve varies with the value of G . It will also depend on the value of G_{XY} relative to G_Y and G_X .

Experimental results

Crouzon syndrome with acanthosis nigricans, an autosomal dominant disorder characterized by craniosynostosis (premature ossification of the skull) and “rough” skin, has been linked to a Ala391Glu mutation in the TM domain of FGFR3^{15,19}, an RTK that plays an important role in skeletal development¹³. The thermodynamics of homodimer formation in lipid bilayers has been characterized for both wild-type FGFR3 TM domain and the Ala391Glu mutant¹⁰. The free energies of homodimerization have been determined as $G_{WT} = -2.8 \pm 0.1$ and $G_M = -4.1 \pm 0.2$ kcal/mole¹⁰.

In heterozygotes, both wild-type and mutant proteins are present, and thus heterodimers are likely to form. The likelihood for heterodimer formation is determined by the free energy of heterodimerization, which is currently unknown. Here we measure FRET between donor-labeled wild-type FGFR3 TM domains and acceptor-labeled Ala391Glu mutants, and we determine the free energy of heterodimerization $G_{WT/M}$ using the analysis presented above.

Wild-type and mutant FGFR3 TM domains (TM_{WT} and TM_M, see figure 2A for the sequences) were produced using solid phase peptide synthesis as described^{20,21}, and purified using reverse phase HPLC. TM_{WT} was labeled with BODIPY-fluorescein (BF), and TM_M was labeled with rhodamine (Rh). The BODIPY-fluorescein/rhodamine pair has been shown to be a suitable FRET pair for such measurements, with a Forster radius $R_0 = 56$ Å¹⁰. The dyes were attached to Cys396, a naturally occurring Cys in the TM domain of FGFR3; this attachment has been shown to not perturb the helicity and the dimerization propensity of FGFR3 TM domain^{8,20}. The labeling yields were determined by comparing dye concentrations (derived from absorbance measurements) and peptide concentrations (determined by CD) as $f_D = 0.86$ and $f_A = 0.73$. To measure FRET, the excitation wavelength was set at 439 nm, and emission spectra were collected from 450 nm to 800 nm. FRET was measured in liposomes containing known concentrations of donor- and acceptor-labeled proteins as previously described in detail elsewhere¹⁷. As an example, Figure 2B shows the spectra of 0.1 mole % BF-TM_{WT} and 0.1 mole % Rh-TM_M (solid line), as well as 0.1 mole % BF-TM_{WT} (dashed line) and 0.1 mole % Rh-TM_M (dotted line). The efficiency of energy transfer, E_{exp} , was calculated from the decrease in donor intensity at 515 nm in the presence of the acceptor (Eq. (5)).

Spectra, similar to the one in Figure 2B, were obtained for various total peptide concentrations in the concentration range from 0.1 to 0.8 mole %, for acceptor-to-donor ratios of 1 (i.e. $[T_d] = [T_a]$). This concentration range is optimal for FRET measurements in liposomes since (i) the proximity contribution to the measured FRET signal is relatively low, (ii) the contribution of scattering to the fluorescence signal is negligible, and (iii) the fluorescence signal amplitude is relatively large¹⁷. Four different experiments were performed for a given total peptide concentration in order to obtain the average values and the standard deviations for the measured FRET efficiencies.

To obtain the FRET efficiencies due to sequence-specific dimerization, E_{dimer} , we first modeled FRET that arises due to random co-localization of donors and acceptors, $E_{proximity}$, according to the analysis of Wolber and Hudson²² using $R_0 = 56$ Å, as described in detail elsewhere^{10,17}. Then, we subtracted the predicted FRET efficiency due to proximity, $E_{proximity}$, from the measured FRET signal E_{exp} to obtain the FRET efficiency due to sequence-specific dimerization E_{dimer} . Next, we followed the protocol outlined above to determine all parameters describing the monomer – dimer equilibrium, namely $[XX]$, $[XY]$, $[YY]$, $[X]$ and $[Y]$, as well as the free energies of heterodimerization $G_{WT/M}$ and the total dimer concentration $[D]$.

Table 1 shows the calculated parameters for 0.1 mole % BF-TM_{WT} and 0.1mole % Rh-TM_M (the experiment shown in Figure 2B). Similar calculations were carried out for all experiments (19 in total). Nineteen values for the free energy of heterodimerization G_{WTM} were determined, and their average and standard deviation were calculated as $G_{WTM} = -3.37 \pm 0.25$ kcal/mol. Comparison of this value to the homodimerization free energies, $G_{WT} = -2.8 \pm 0.2$ kcal/mol and $G_M = -4.1 \pm 0.2$ kcal/mol, reveals that G_{WTM} is the average of G_{WT} and G_M .

The side chain of Glu391 has hydrogen bonding capabilities, and it has been proposed that the mutant dimer is stabilized via hydrogen bonding interactions between Glu391 and the adjacent helix in the dimer, without changing the dimer interface¹⁰ and therefore the orientation of the catalytic domains²³. It is not clear, however, whether two, or just one, hydrogen bonds form in the mutant homodimer. Here we find that the Glu391 contribution to the energetics of heterodimerization (-0.6 kcal/mole) is half the difference between wild-type and mutant free energy of homodimerization (-1.3 kcal/mole). This finding may be indicative that the mutant homodimer is stabilized by two hydrogen bonds, while a single hydrogen bond forms in the heterodimer. The exact hydrogen bonding pattern in the mutant homodimer and the heterodimer should emerge from solid state NMR studies, currently underway in our laboratory.

In Figure 3A we compare the total dimer fractions for TM_{WT} (dashed line), TM_M (dotted line) and for the equimolar mixture of TM_{WT} and TM_M (open circles and solid line), as a function of total protein concentration. When only wild-type proteins are present, the dimer fraction is determined solely by the value of G_{WT} . In the equimolar mixture, the total dimer concentration is a sum of wild-type homodimers, mutant homodimers, and heterodimers, (i.e. $[D] = [TM_{WT}TM_{WT}] + [TM_MTM_M] + [TM_{WT}TM_M]$), and is thus determined by the three values, G_{WT} , G_M and G_{WTM} .

Figure 3B shows the results for TM_{WT} (dotted line) and TM_{WT}/TM_M, (dashed line), re-plotted on a semi-logarithmic scale, and thus covering a much wider concentration range. The subtraction of the two lines gives the increase in total dimer fraction (solid line) due to the substitution of 50% of the wild-type with mutant proteins. As discussed previously, the structure of all FGFR3 TM dimers are expected to be similar, with Glu391-mediated hydrogen bond(s) enhancing stability but not inducing a large structural perturbation¹⁰. Therefore, the relative increase in total dimer fraction (i.e. difference in dimer fraction (solid line), as compared to the wild-type dimer fraction (dotted line)) should correlate with pathology induction due to the Ala391Glu mutation in heterozygotes. We see that the difference in total dimer fraction for low peptide concentrations (lower than 0.1 mole%) exceeds the wild-type dimer fraction and thus can significantly affect the monomer-dimer equilibrium. Therefore, substitution of 50% of the wild-type with mutant proteins could increase the total (hetero- plus homo-) dimer fraction in a biologically significant manner under certain conditions¹⁰. This increase is a likely determinant of pathology induction in Crouzon syndrome with acanthosis nigricans in heterozygotes.

CONCLUSION

Here we present a method to characterize the energetics of heterodimerization in lipid bilayers using FRET. We use it to determine the propensity for heterodimer formation between wild-type FGFR3 TM domain and the Ala391Glu mutant, linked to the autosomal dominant Crouzon syndrome with acanthosis nigricans and to bladder cancer. Furthermore, we predict changes in the over-all monomer-dimer equilibrium due to the mutation in heterozygotes. TM domain mutations in FGFR3 and other FGFR family members are known to induce various autosomal dominant skeletal disorders, such as achondroplasia and

hypochondroplasia^{14,24}. Therefore, the presented method to quantify heterodimer energetics should have a broad utility in studies of pathology induction mechanisms, as well as in studies of the general membrane protein folding principles.

Acknowledgments

We thank Dr. Xue Han for technical assistance and Dr. W. C. Wimley for reading the manuscript prior to publication. This work was supported by Research Scholar Grant # RSG-04-201-01 from the American Cancer Society to K.H. and, in part, by NSF MCB-0315663 to K.H.

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A.

B

Figure 1.

(A) Schematic view of a lipid bilayer with two different TM helical species, X and Y, associating into three types of dimers: the homodimers XX and YY and the heterodimer XY.

(B) Equilibrium curves (total dimer fraction versus total peptide concentration) for homo- and heterodimerization. The solid line shows the total dimer fraction for a homodimeric case, with free energy of homodimerization $G = -3.4 \text{ kcal/mole}$. The dashed and the dotted curves show the total dimer fraction $[D]/[T]$ when helices X and Y are both present, such that the homodimers XX and YY, and heterodimers XY form. The dashed line describes the case in which X and Y have similar dimerization propensities ($G = G_Y - G_X$ is small).

The dotted line describes the case in which X and Y have very different dimerization propensities ($G = G_Y - G_X$ is large). For the heterodimeric case, the total dimer concentration is $[D] = [XX] + [YY] + [XY]$ and we have assumed $[T_X] = [T_Y]$, $G_{XY} = G$; $G_X = G - G/2$, $G_Y = G + G/2$, where $G = -1$ and -5 kcal/mole, respectively, for the dashed and the dotted line. The concentrations $[XX]$, $[YY]$, and $[XY]$ can be calculated by solving equations (2), (3), and (4). As G increases, the shape of the equilibrium curve for the heterodimer will increasingly deviate from the homodimeric curve. For a given G , the shape of the equilibrium curve will depend on the value of G_{XY} and on the $[T_X]/[T_Y]$ ratio.

A

B

Figure 2.

(A) Amino acid sequences of the two peptides used in this study, TM_{WT} and TM_M (see ¹⁰ for details). The predicted hydrocarbon core-embedded segments, identified using hydropathy analysis ²⁵, are underlined. The Ala391 Glu mutation, shown in bold, has been linked to Crouzon syndrome with acanthosis nigricans ¹⁵. It has also been identified as a somatic mutation in bladder cancer ¹⁶.

(B) Fluorescence emission spectra of BODIPY-Fluorescein (BF)/Rhodamine (Rh) - labeled heterodimers in POPC vesicles. Solid line: 0.1mole % BF-TM_{WT} and 0.1mole % Rh-TM_M. Dashed line: 0.1 mole % BF-TM_{WT}. Dotted line: 0.1mole % Rh-TM_M. Labeling yield was

$f_D = 0.86$ and $f_A = 0.73$ for the donor and the acceptor, respectively; the total peptide concentration was therefore 0.25 mole %. In the experiments, labeled peptides were premixed with POPC lipids in organic solvent, the solvent was evaporated, and the samples were hydrated and freeze-thawed three times to achieve equilibrium, as described in detail elsewhere^{8,17}. The excitation was fixed at 439 nm, such that only BODIPY-Fl was directly excited. The FRET efficiency was calculated from the decrease in BODIPY-Fl fluorescence at 515 nm (Eq. 5).

A

B

Figure 3.

TM_{WT}/TM_M dimerization in POPC vesicles. **(A)** Total dimer fraction as a function of total peptide concentration, plotted on a linear scale. Open circles: total dimer fraction for TM_{WT}/TM_M at the studied peptide concentrations, calculated from the measured FRET efficiencies using Eqs. (11), (12), (13), and (2). Solid line: total dimer fraction for TM_{WT}/TM_M , calculated from Eqs. (2), (3), and (4) for $G_{WT/M} = -3.37$ kcal/mol. Dotted line: homodimeric dimer fraction for TM_{WT} (results from ¹⁰). Dashed line: homodimeric dimer fraction for TM_M (results from ¹⁰). **(B)** Total dimer fractions for TM_{WT}/TM_M (dashed line) and TM_{WT} (dotted line), re-plotted on a semi-logarithmic scale. (Note, while the protein

concentration range in Figure 3A has been shown to be optimal for FRET measurements^{8,17}, the plot in Figure 3B covers a much wider concentration range). The solid line, obtained via subtraction, is the change in total dimer fraction due to the substitution of 50% of the wild-type with mutant proteins.

Table 1

Monomer-dimer equilibrium parameters, calculated from the experimental spectra shown in Figure 2. All concentrations are in dimensionless units, moles of peptide per moles of lipid¹⁷.

F_D , counts/s	220920
F_{DA} , counts/s	165740
E_{meas} , Eq. (5)	0.2498
$E_{proximity}$, Ref.[^{10,17}]	0.1467
E_{dimer} , Eq. (6)	0.1031
$[T_X]$, Eq. (8)	0.00116
$[T_Y]$, Eq. (8)	0.00137
$[XY]$, Eq. (11)	0.00016
$[XX]$, Eqs.(12) and (2)	0.00008
$[YY]$, Eqs.(12) and (2)	0.00032
$[D] = [XX] + [YY] + [XY]$	0.00056
$[X]$, Eq. (12)	0.00084
$[Y]$, Eq. (13)	0.00057
K_{XY} , Eq. (14)	339
G_{XY} , kcal/mol, Eq. (15)	-3.48 kcal/mole