

# The Stability of Transmembrane Helix Interactions Measured in a Biological Membrane

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Despite some promising progress in the understanding of membrane protein folding and assembly, there is little experimental information regarding the thermodynamic stability of transmembrane helix interactions and even less on the stability of transmembrane helix–helix interactions in a biological membrane.

Here we describe an approach that allows quantitative measurement of transmembrane helix interactions in a biological membrane, and calculation of changes in the interaction free energy resulting from substitution of single amino acids. Dimerization of several variants of the glycoprotein A transmembrane domain are characterized and compared to the wild-type (wt) glycoprotein A transmembrane helix dimerization. The calculated  $\Delta\Delta G^{\text{app}}$  values are further compared with values found in the literature. In addition, we compare interactions between the wt glycoprotein A transmembrane domain and helices in which critical glycine residues are replaced by alanine or serine, respectively. The data demonstrate that replacement of the glycine residues by serine is less destabilizing than replacement by alanine with a  $\Delta\Delta G^{\text{app}}$  value of about 0.4 kcal/mol.

Our study comprises the first measurement of a transmembrane helix interaction in a biological membrane, and we are optimistic that it can be further developed and applied.

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## Introduction

The initial folding of  $\alpha$ -helical membrane proteins can be conceptualized as a two-stage process.<sup>1</sup> In stage I, hydrophobic polypeptide domains integrate individually into a membrane and form transbilayer helices. Individual transmembrane helices can therefore be considered as independent folding domains. In a second stage, individual helices interact side-to-side, resulting in a transmembrane helix bundle. Subsequent events refine on this structure, placing prosthetic groups and re-entrant loops into the helix bundle.<sup>2</sup> Specific interactions between helices are largely driven and stabilized by defined packing between two helices and by

hydrogen bonding.<sup>3–5</sup> Other factors such as lipid–lipid and protein–lipid interactions may also play a role. One of the best studied examples for stage II of the two-stage-model is the homo-dimerization of the glycoprotein A (GpA) transmembrane helix. GpA is a transmembrane protein present on the surface of red blood cells and consists of a large extracellular domain, a transmembrane region, and a small cytoplasmic segment. Several studies have highlighted the importance of the LxxGVxxGVxxT motif for dimerization of GpA, and especially the GxxxG motif was shown to be important for mediating and stabilizing the transmembrane homo-dimer.<sup>6–11</sup> Besides characterization of the GpA dimerization and identification of critical residues, GpA was also studied in terms of energetic contributions from individual side-chains for interaction of the GpA transmembrane helix. The energetics of GpA dimerization was analyzed by Förster resonance energy transfer (FRET) measurements in various detergents and the free energy for dimerization was found to vary between

Abbreviations used: wt, wild-type; GpA, glycoprotein A; FRET, Förster resonance energy transfer; MBP, maltose binding protein; TM, transmembrane.

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3.7 and 7.5 kcal/mol.<sup>12,13</sup> These data indicate that interaction of transmembrane domains is not only influenced by the amino acid sequence of the peptide but also by the lipid or detergent environment. GpA homo-dimerization was studied more thoroughly by analytical ultracentrifugation, which allows measurement of the dissociation constant of interacting helices. Using this approach, K. Fleming and co-workers have characterized numerous GpA variants with single and multiple amino acid substitutions.<sup>14–17</sup> Interestingly, it was also shown by this method that the detergent influences the strength of the GpA interaction, but that the hierarchy in the interaction strength of different mutants did not change in various detergents,<sup>16</sup> revealing that the specific helix–helix interaction is relatively independent from environmental effects. Besides FRET and analytical ultracentrifugation, small angle X-ray scattering was also used to determine the dissociation constant for a GpA transmembrane helix mutant.<sup>18</sup>

A simple approach to measure the stability of polytopic membrane proteins *in vitro* has been described recently, in which a membrane protein is solubilized in a non-ionic detergent and then denatured by titrating in the anionic detergent sodium dodecyl sulfate (SDS).<sup>19,20</sup> While the denatured state contains almost as much secondary structure as the native state, the individual  $\alpha$ -helices are most likely isolated from each other and lack tertiary contacts. Thus, this method measures in effect the total strength of interhelical associations, i.e. stage II in the two-stage-model.

Nevertheless, there is little experimental information regarding the thermodynamic stability of transmembrane helix interactions and even less on the stability of transmembrane helix–helix interactions in a biological membrane.

In recent years, genetic systems have been developed that allow measurement of the interaction of single transmembrane helices in a biological membrane.<sup>8,9,21,22</sup> In these systems a transmembrane helix of interest is genetically fused to a DNA binding domain and expressed across a bacterial membrane. Oligomerization of the transmembrane helix brings the DNA binding domains close together, facilitating binding to a promoter/operator region on the DNA. Binding to the promoter/operator controls the expression of a reporter gene and therefore the relative strength of a transmembrane helix–helix interaction can be determined by measuring the activity of the reporter.

Here we use the GALLEX system, which allows measurement of the interaction of single transmembrane helices in the *Escherichia coli* inner membrane,<sup>21,23,24</sup> to estimate the consequences of single amino acid substitutions on the free energy of dimerization in a biological membrane. Several GpA variants are characterized, and dimerization is compared to the wild-type (wt) GpA transmembrane helix. The calculated  $\Delta\Delta G^{\text{APP}}$  values are compared with values found in the literature.

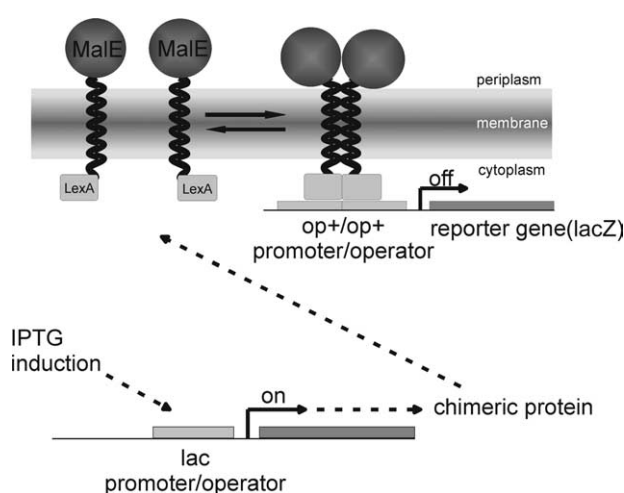
Additionally, we found that replacement of critical Gly residues by Ser is less destabilizing than replacement by Ala with a  $\Delta\Delta G$  value of about 0.4 kcal/mol.

Our study comprises the first measurement of a transmembrane helix interaction in a biological membrane. The approach should be applicable to other transmembrane helix–helix interactions.

## Results

### GALLEX quantitative

Several genetic systems have been developed that allow observation of the interaction of single transmembrane helices in the inner membrane of *E. coli*.<sup>8,9,21,22</sup> In the GALLEX system (Figure 1) a transmembrane helix of interest is genetically fused to the DNA binding domain of the *E. coli* LexA protein. After expression of the chimeric protein, the protein integrates into the *E. coli* inner membrane. Transmembrane helix dimerization is reported by binding of the homo-dimeric LexA protein to a promoter/operator region located in the *E. coli* genome. This operator regulates the expression of  $\beta$ -galactosidase, and binding of the homo-dimeric LexA DNA-binding domain represses transcription of the *lacZ* gene, which results in reduced activity of the reporter. Thus, the activity of the reporter ( $\beta$ -galactosidase) gives a measure of the relative strength of a transmembrane helix–helix interaction. Nevertheless, this assay only allows a comparison of relative strength rather than providing absolute numbers.

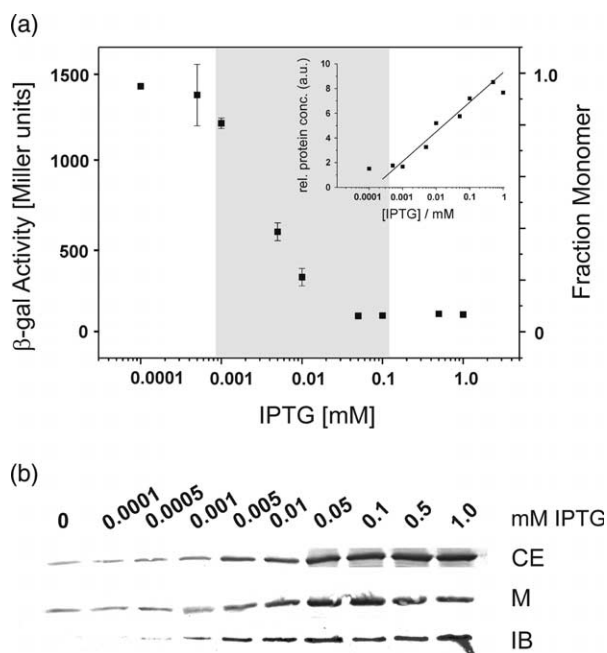


**Figure 1.** The GALLEX system. Addition of IPTG induces expression of the chimeric proteins. After membrane integration of the proteins, dimerization of the LexA DNA-binding domain is mediated by oligomerization of the transmembrane helices. The homo-dimeric LexA DNA-binding domain can bind to the op+ promoter/operator and binding results in repression of the reporter gene activity (*lacZ*).

In the GALLEX system an inducible *lac* promoter controls the expression of the chimeric protein. The *lac* promoter can be gradually induced, which allows the expression level of a protein to be progressively controlled.

To test whether the GALLEX system can be used to control the expression of a chimeric protein with a transmembrane domain, the GpA wt transmembrane domain was genetically fused to the LexA DNA-binding domain and  $\beta$ -galactosidase activity was measured at various IPTG concentrations (Figure 2(a)). In Figure 2(b) the expression level of a chimeric protein is shown at various IPTG concentrations used for induction of the *lac* promoter, and in the range between 0.001 to 0.5 mM IPTG a linear correspondence between the total protein expression level and the IPTG concentration is given (inset in Figure 2(a)).

Increasing the IPTG concentration leads to a gradual increase in the amount of expressed chimeric protein. At IPTG concentrations >0.5 mM the *lac* promoter is completely induced and no further significant decrease of the  $\beta$ -galactosidase activity was observed. As can be seen in



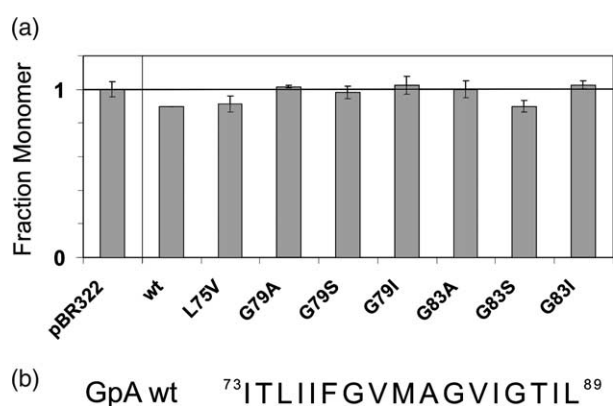
**Figure 2.** Dimerization of GpA wt transmembrane helix in a biological membrane.  $\beta$ -Galactosidase activities (left axis) were measured in *E. coli* SU101 after induction of protein expression using various concentrations of IPTG. The relative protein concentration at various IPTG concentrations (inset) was determined by densitometric analysis of the Western blot shown in (b). Since even at lower IPTG concentrations a significant fraction of the wt GpA transmembrane helix dimerizes, the value of 100% fraction monomer (right axis) was determined as described in the legend to Figure 3 and in the text. In (b) the expression of the chimeric proteins at the various IPTG concentrations is shown using whole cell extract (CE), membranes (M), or the fraction containing inclusion bodies (IB). Expression was probed using anti-LexA antibodies (Invitrogen).

Figure 2(b), some protein is expressed even at 0 mM IPTG. In addition, we observed that with increasing IPTG concentrations a significant fraction of the expressed chimeric protein is deposited in inclusion bodies rather than incorporated into the *E. coli* inner membrane (Figure 2(b)). While misfolded proteins deposited in inclusion bodies are not expected to interfere with the assay, the effective amount of protein incorporated into the *E. coli* membrane is only linear in the range between 0.001 and 0.1 mM IPTG (as indicated in Figure 2). At low IPTG concentration the wt GpA transmembrane helix does not reach the 100% monomer level, but it does reach almost a plateau at an IPTG concentration of 0.05 mM, and at higher IPTG concentrations the  $\beta$ -galactosidase activity does not change significantly anymore. Although there is no linear correlation between [IPTG] and the amount of membrane incorporated protein at higher IPTG concentrations, the effective amount of expressed protein still increases at [IPTG] > 0.1 mM, whereas the amount of membrane incorporated protein does not, and subsequently the  $\beta$ -galactosidase activity does not increase.

To define the “100% monomer” level, the  $\beta$ -galactosidase activity of a strain containing an empty expression plasmid (pBR322) was measured. On this plasmid no LexA DNA-binding domain is encoded, and, therefore, the *op+* promoter/operator in *E. coli* SU101 is 100% active. The measured  $\beta$ -galactosidase activity represents the activity at 0% repression. In Figure 3 the relative  $\beta$ -galactosidase activity obtained using a strain transformed with the empty plasmid is shown as well as  $\beta$ -galactosidase activities of strains transformed with various plasmids used in this study. As can be seen in Figure 3, in strains where a GpA variant with an Ala or Ile substituted Gly residue is expressed, the  $\beta$ -galactosidase activities reach the level of the control strain, indicating that these transmembrane helices are monomeric.

### Calculating the free energy differences of GpA variants

The titration data shown in Figure 2 have a sigmoid appearance, which could, in the simplest case, represent dissociation of the GpA monomer and, therefore, could be fitted assuming a simple monomer–dimer equilibrium. In recent years, a vast majority of data have proven that GpA is in a monomer–dimer equilibrium in various environments. Nevertheless, in a biological system many equilibria have to be considered. In addition to the association/dissociation of the GpA dimer, the steepness of the curve is the sum of several equilibria and simplified assumptions have to be applied with caution. The use of a genetic system for studying oligomerization of transmembrane domains is for example complicated by the fact that increased surface density of the transmembrane domains in the membrane does not only affect the monomer–dimer equilibrium but



**Figure 3.** Comparison of  $\beta$ -galactosidase activities at 0 mM IPTG. *E. coli* SU101 was transformed with the empty expression plasmid pBR322 as well as with pBLM plasmids<sup>21,24</sup> encoding different GpA transmembrane helix variants used in this study. Expression of proteins was not induced in the cultures and the  $\beta$ -galactosidase activity was measured as described in Materials and Methods. The  $\beta$ -galactosidase activity of the strain containing the empty vector was set as 1 and all other values are shown relative to this. Error bars represent data combined from three independent measurements. In (b) the sequence of the wt GpA transmembrane domain used for the GALLEX measurements is given.

also the absolute amount of active LexA whose dimeric form is able to repress the reporter gene activity. The application of a simple monomer-dimer model, which neglects other biologically relevant equilibria, will of necessity be incomplete. However, a complete deconvolution of the titration curve requires knowledge of all involved equilibria and their respective dissociation constants. In the absence of this knowledge, we have to limit ourselves to the monomer-dimer model. Nevertheless, the good correspondence between our results and the known stabilities of the different GpA variants is reassuring.

To obtain relative values for the dimerization propensities of the different mutants, we first converted activity to fraction monomer under the assumption that full activity corresponds to 100% monomer and that activity is linearly correlated with fraction monomer. We then plotted fraction monomer *versus* [IPTG] and interpolated from the graph the [IPTG], at which the fraction monomer is 0.5. This [IPTG]<sub>50%</sub> was considered a first approximation to an apparent dissociation constant  $K_D$  (assuming a linear relationship between [IPTG] and the GpA concentration), from which the free energy of dimerization  $\Delta G_{\text{dim}}$  was calculated using the relationship  $\Delta G_{\text{dim}} = -RT \ln(K_D)$ .

We have measured the interaction tendency of three GpA mutants and compared the obtained data to the interaction of the wt transmembrane domain (Table 1). Integration of the chimeric proteins into the *E. coli* inner membrane was confirmed by cellular fractionation (compare

**Table 1.** Free energy perturbations caused by amino acid substitutions at the helix interface of the GpA TM

GpA	$K_D^{\text{app}}$ ( $\mu\text{M}$ )	$\Delta G^{\text{app}}$ (kcal/mol)	$\Delta\Delta G^{\text{app}}$ (kcal/mol)
wt	3.1	7.51	0.00
L75V	4.6	7.27	0.23
G79I	47	5.90	1.61
G83I	118	5.35	2.15

Titration curves, as shown in Figure 2, have been used to determine apparent  $K_D$  values for the GpA transmembrane helices with amino acid substitutions. Apparent  $K_D$  values were determined as described in the text.  $\Delta G^{\text{app}}$  was calculated according to  $\Delta G^{\text{app}} = -RT \ln K_D$ , with  $K_D = [M]^2/[D]$ . For estimating the  $\Delta\Delta G$  values, changes in the apparent  $\Delta G$  values of the GpA TM variants relative to the wt were calculated.

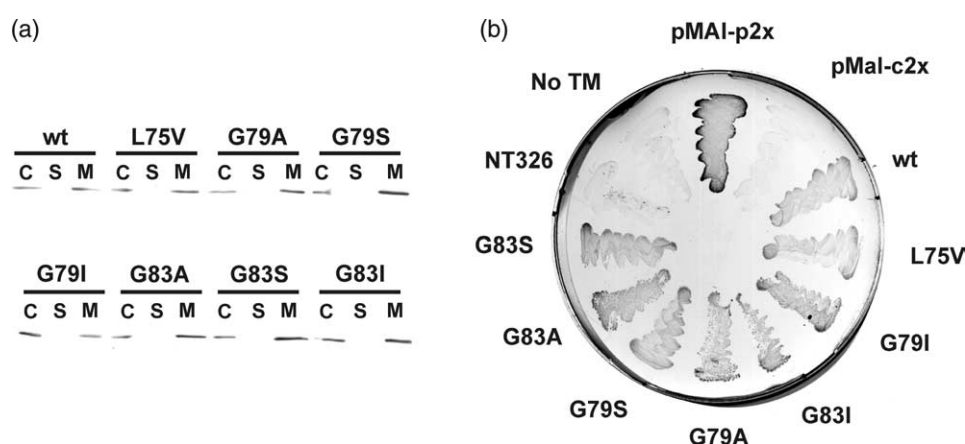
Figure 2) or alkali extraction, and the maltose binding protein (MBP) complementation assay confirmed that all fusion proteins were inserted with the right topology (Figure 4). It has been shown that replacement of either G79 or G83 by Ile results in a strong decrease of interaction, whereas substitution of L75 by Val only weakens the dimeric interaction slightly.<sup>6,8,9</sup> The expression level for all constructs used in this study was tested by Western blot analysis using an antibody directed against the LexA domain. For all expressed proteins similar expression levels were found (data not shown). After calculation of the apparent  $\Delta G$  values, the apparent free energies of the mutated GpA transmembrane helices were compared to the wt helix (Table 1). The results show that replacing Gly79 or Gly83 by Ile in the GpA transmembrane helix destabilizes the helix-helix interaction and the change of the free energy  $\Delta\Delta G^{\text{app}}$  is about 1.6 kcal/mol or 2.15 kcal/mol, respectively. Substitution of L75 weakens the helix-helix interaction and the change of the free energy  $\Delta\Delta G^{\text{app}}$  is only about 0.23 kcal/mol.

#### How much can a Gly $\rightarrow$ Ser substitution contribute to the stability of a transmembrane helix-helix interaction?

We have recently shown that replacement of the GpA G79 and G83 residues by Ala greatly weakens the transmembrane helix-helix interaction whereas a replacement of either Gly by Ser results in moderately strong GpA homo-dimerization.<sup>24</sup>

This stabilization was discussed to be most likely caused by electrostatic interactions between the Ser side-chain  $-\text{OH}$  and a backbone carbonyl oxygen atom on the adjacent helix.<sup>24</sup>

To estimate how much such an interaction can stabilize a transmembrane helix dimer in a biological membrane the interaction tendency was measured for the GpA wt transmembrane helix as well as for the G79A, G79S, G83A, G83S substituted helices. The  $\beta$ -galactosidase activities were then used to determine the apparent  $K_D$  values and subsequently to calculate  $\Delta\Delta G^{\text{app}}$  for the individual transmembrane domains (Table 2). Replacement of G79 or G83 by Ala



**Figure 4.** Test for insertion and orientation of the expressed chimeric proteins in *E. coli*. (a) Western analysis of *E. coli* cell extracts after NaOH extraction. C, whole cells; S, supernatant after NaOH extraction (soluble proteins); M, pellet after NaOH extraction (membrane proteins). The expressed chimeric proteins with a molecular mass of 54 kDa were found solely in the membrane protein fraction (pellet). For details see Materials and Methods. (b) Maltose binding protein complementation assay to test for LexA(TM)MBP orientation. *E. coli* NT326 cells were transformed with various constructs and cultivated on M9 agar containing 0.4% (w/v) maltose and 0.02% (w/v) IPTG. The GpA TM sequences inserted were wt, L75V, G79A, G79S, G79I, G83A, G83S, and G83I. The MBP expression from pMAL-p2x results in periplasmic location of the MBP domain while after expression from pMAL-c2x the MBP is located in the cytoplasm.

destabilized the helix dimer and the changes of the free energy  $\Delta\Delta G^{\text{aPP}}$  were 1.2 kcal/mol and 1.0 kcal/mol, respectively. Replacement of the Gly residues by Ser only slightly weakens the interaction ( $\Delta\Delta G^{\text{aPP}} = 0.36$  kcal/mol (G79S) or 0.32 kcal/mol (G83S)). The only difference between Ala and Ser is the presence of an OH group at the C<sup>β</sup> carbon atom. In terms of packing effects, Ser should favor tight packing even less than Ala and the stabilization of the helix dimer is most likely caused by electrostatic interactions, as discussed recently.<sup>24</sup> Comparing the  $\Delta\Delta G^{\text{aPP}}$  values for the Ala substituted and the Ser substituted helices suggests that the electrostatic interactions can stabilize the helix dimer by about 0.4 kcal/mol (the observed differences are about 0.8 kcal/mol (Table 2), but two hydrogen bonds have to be considered in the dimer).

## Discussion

### Energetics of transmembrane helix interactions

About 15 years ago it was hypothesized that the formation of individual transmembrane helices can

**Table 2.** Free energy perturbations caused after substitution of two critical glycine residues by alanine or serine

GpA	$K_D^{\text{aPP}}$ ( $\mu\text{M}$ )	$\Delta G^{\text{aPP}}$ (kcal/mol)	$\Delta\Delta G^{\text{aPP}}$ (kcal/mol)
wt	3.1	7.51	0.00
G79A	23.7	6.30	1.20
G79S	5.7	7.15	0.36
G83A	16.9	6.50	1.00
G83S	5.3	7.19	0.32

Apparent  $K_D$  values were determined as described in the text. For details see the legend to Table 1.

be energetically separated from subsequent assembly into helical bundles.<sup>1</sup> After membrane insertion of individual  $\alpha$ -helical transmembrane helices (stage I) several helices interact and form higher ordered oligomeric structures (stage II). Homo-dimerization of the GpA transmembrane helix has been proven to be a useful example for studies of helix interaction. The protein forms independently stable transmembrane  $\alpha$ -helices, and homo-dimerization of the transmembrane domain can be investigated in various environments. Several methods have been applied that allow comparison of the relative interaction tendency of the wt GpA transmembrane helix and helices with single or multiple amino acid substitutions. Förster resonance energy transfer (FRET) and analytical ultracentrifugation have allowed measurement of dissociation constants of the GpA transmembrane helix in detergents and calculation of the free energy of association/dissociation<sup>12–17</sup>. While analytical ultracentrifugation has provided excellent measures of interaction energies for numerous point mutated GpA transmembrane helices, the experiments must be done in a detergent environment rather than in a biological membrane. Even though the stability of GpA sequence variants varies in different environments, the hierarchy of stability for sequence variants is largely conserved.<sup>16</sup> This observation suggests that the energy of association/dissociation can most likely be separated from contributions arising from protein–lipid and lipid–lipid energy terms. Although these considerations encourage the study of transmembrane helix interactions in micelles or vesicles, it remains desirable to measure transmembrane helix interactions in their natural environment: a biological membrane. The approach described here allows quantitative measurement of

transmembrane helix interactions in a biological membrane, and estimation of changes in the interaction free energy resulting from substitution of single amino acids.

### Using genetic systems to measure energetics of transmembrane helix interactions

Expression of the protein in the GALLEX assay is driven by the *lac* promoter from *E. coli*, which allows controlling the expression level by varying the amount of the inductor (IPTG) in the growth medium. A gradual increase of the IPTG concentration leads to a gradual increase of protein expression in the range between 0.001 and 0.5 mM IPTG (Figure 2), but the measured  $\beta$ -galactosidase activities are not linear with the IPTG concentration. As can be seen in Figure 2(b), at [IPTG] > 0.1 mM the amount of membrane incorporated fusion protein does not increase gradually anymore. Therefore, only in the range between 0.001–0.1 mM IPTG is the amount of membrane incorporated chimeric protein directly proportional to the IPTG concentration. At high IPTG concentrations (> 0.5 mM) no further increase in the protein expression is observed, most likely due to complete induction of the promoter. At 0 mM IPTG, expression from the *lac* promoter is not completely abolished, as can be seen in Figure 2(b). This background expression explains why the GpA wt transmembrane helix does not reach the same  $\beta$ -galactosidase activities at low IPTG concentrations as other mutants do, e.g. the G83I mutant (Figure 3). Even at the low protein concentrations, a significant amount of the wt GpA transmembrane helix (~10%) dimerizes. Therefore, to define the “100% monomer” value the  $\beta$ -galactosidase activity was measured using a strain transformed with an empty plasmid, since in this strain the *op+* promoter is completely active (Figure 3).

### Determination of the apparent $K_D$ values allows calculation of $\Delta G^{\text{app}}$

The results show that replacement of G79 or G83 by Ile weakens the helix–helix interactions, and the changes of the free energy  $\Delta\Delta G^{\text{app}}$  are about 1.6 and 2.1 kcal/mol, respectively. Replacement of L75 by Val only costs about 0.23 kcal/mol (Table 1). The observed values are about 1.5 kcal/mol lower than those determined recently by analytical ultracentrifugation.<sup>15</sup> One possible explanation for this observed difference is that different chimeric proteins were used in the two studies. In the report by Doura *et al.*<sup>15</sup> the GpA transmembrane helix was fused to nuclease A from *Staphylococcus aureus* at the N terminus of the transmembrane domain. In our study the GpA transmembrane domain was fused to the MalE domain as well as to the LexA DNA-binding domain. It is possible that the fusion partners might influence the strength of a given interaction.

After fusion of the wt GpA transmembrane domain to nuclease A, a free energy of dissociation

of 9 kcal/mol was found using analytical ultracentrifugation.<sup>17</sup> FRET analysis with chemically labeled GpA peptides revealed a dissociation free energy in the range from 3.7 kcal/mol to 7.5 kcal/mol, depending on the detergent system used.<sup>12,13</sup> These results suggest that both the detergent and the fusion construct might influence a transmembrane helix interaction. In addition, in the report by Doura *et al.*<sup>15</sup> 23 amino acids of the GpA transmembrane domain were analyzed whereas in this study only 17 amino acids were fused to the LexA DNA-binding domain (see Figure 3(b)).

In contrast to *in vitro* measurements with isolated peptides or fusion proteins, in a biological system like *E. coli*, more equilibria than just the monomer–dimer equilibrium have to be considered for analysis, which does not allow interpretation of the data assuming a simple monomer–dimer model. But since we only compare the strength of interactions between different transmembrane helices and the measurements were carried out under exactly the same experimental conditions, the measured differences must directly result from the amino acid substitutions within the GpA transmembrane regions. As mentioned above, the free energy of interaction varies with the environment and therefore the simplest explanation for differences observed between the studies is that we measure dimerization in a biological membrane rather than in detergent.

### Gly-Ser substitutions result in a decrease of the free energy of dissociation

We used our approach to test how much replacement of critical Gly residues by Ala or Ser, respectively, weakens a transmembrane helix interaction. While single Ser residues in synthetic peptides were found not to stabilize transmembrane helix interactions significantly *in vitro*,<sup>25,26</sup> we recently showed that Ser residues can stabilize a helix dimer if surrounding residues mediate and stabilize dimerization.<sup>24</sup> The difference in the strength of interaction between the Ala and Ser substituted Gly residues (Table 2) cannot be explained by packing effects. Ser has an even bulkier side-chain than Ala, which should disfavor tight packing more strongly. In our previous work we have shown that replacement of either Gly residue by Ser most likely results in electrostatic interactions between the Ser OH side-chain and a backbone carbonyl on the adjacent helix.<sup>24</sup> But how much can such an interaction contribute to the stability of a transmembrane helix dimer? To estimate the potential energetic contribution of electrostatics for mediating and stabilizing a transmembrane helix interaction, we have analyzed GpA variants where the Gly residues at positions 79 and 83 are replaced by Ala or Ser. While Ala disfavors close packing and therefore destabilizes the helix–helix interaction ( $\Delta\Delta G^{\text{app}} = 1.2$  kcal/mol and 1.0 kcal/mol, respectively), substitution of either Gly residue by Ser results in lesser destabilization of the helix dimer ( $\Delta\Delta G^{\text{app}} = 0.36$  kcal/mol and

0.32 kcal/mol, respectively). Comparing the values obtained with the Ala and the Ser substituted GpA transmembrane helices suggests that formation of a hydrogen bond stabilizes the dimer by about 0.4 kcal/mol. This value is in good agreement with data obtained recently by analytical ultracentrifugation. T87 of the wt GpA transmembrane domain was shown to form a hydrogen bond to a backbone carbonyl on the adjacent helix.<sup>27</sup> Replacement of T87 in the GpA transmembrane helix by Ala results in a decrease of the free energy of dissociation of about 1 kcal/mol (0.5 kcal/mol per monomer). Unfortunately, in this study contributions from hydrogen bond formation cannot be clearly separated from other stabilizing effects, since T87 is naturally present in the GpA transmembrane helix and packing of the helix dimer is optimized for this residue. The use of the two G79 and G83 variants allows separating of effects caused by close packing from stabilizing effects caused by formation of a hydrogen bond. Nevertheless, the agreement is remarkable and indicates that introducing a single Ser residue can stabilize a transmembrane helix interaction with a  $\Delta G$  value in the range of 0.3–0.5 kcal/mol.

## Conclusions

Here we describe for the first time a system to measure changes in the free energy of dissociation in a biological membrane, rather than in cell-free systems. The approach has several advantages over studies in detergent systems: first, transmembrane helix interactions are seen in a biological membrane rather than in a membrane mimicking environment, and the GALLEX system allows controlling the expression level of the transmembrane domain *in vivo*; second, measuring the interaction in living cells is not complicated by chemical synthesis of peptides, by labeling or by purification of synthesized or heterologously expressed proteins and peptides; third, the system is relatively simple and should allow measurement of numerous transmembrane helices in a relatively short time.

So far the approach suffers from the fact that only  $\Delta\Delta G^{\text{aPP}}$  values were determined rather than  $\Delta G$  values. Our efforts to quantify the exact concentration of the transmembrane domain per cell surface area and thus to determine  $\Delta G$  values are not yet convincing. Nevertheless, this is the first time that the stability of a transmembrane helix interaction has been determined in a biological membrane and we are optimistic that it can be further developed and applied.

## Materials and Methods

### GALLEX assay

All plasmids used in this study were made by ligating synthetic oligonucleotide cassettes, which encode the TM sequences of interest, into the SpeI/SacI restriction

digested vector pBLM100.<sup>21</sup> Ligation of the oligonucleotide cassette results in an open reading frame encoding the LexA DNA-binding domain followed by the transmembrane helix and the MalE domain. *E. coli* cells were transformed according to standard procedures, and expression from the construct places the MalE in the periplasm, the helix across the membrane, and the LexA domain in the cytoplasm.

The association capacity of different chimeric proteins is measured as the repression of reporter gene ( $\beta$ -galactosidase) activity in the *E. coli* strain SU101.<sup>21,28</sup> Three independent clones of each transformant were grown overnight in the presence of IPTG. On the next day cells were diluted to an  $A_{600}=0.1$  in LB medium containing the appropriate antibiotics and the indicated concentrations of IPTG. At an  $A_{600}$  of around 0.6 cells were harvested and  $\beta$ -galactosidase activity was measured as described.<sup>29</sup>

### Test for membrane insertion and orientation

Each single plasmid used for the GALLEX measurements was tested for proper integration of the encoded chimeric protein into the *E. coli* inner membrane using cell fractionation, solvent extraction, and complementation. To determine the localization of the expressed chimera, *E. coli* cells were broken by sonification in 20 mM HEPES (pH 7.5), 5 mM EDTA, and inclusion bodies and cell debris were sedimented by centrifugation at 10,000g for 15 min. Inclusion bodies were further purified by washing the resulting sediment three times with buffer containing 1% (v/v) Triton X-100 to remove residual membrane fragments. After the first centrifugation step the supernatant was further centrifuged at 100,000g for 30 min, resulting in sedimentation of membranes.

To prove that the chimeric protein is integrated in the *E. coli* inner membrane, membranes were extracted with 0.05 M NaOH.<sup>30</sup> Only integral proteins remain in the membrane after this treatment. To further ensure that the MBP moiety of the chimera is located in the bacterial periplasm, plasmids were transformed in *E. coli* NT326 cells. In this strain the endogenous *malE* gene is deleted and the cells can only grow on minimal medium containing maltose as the only carbon source if the MBP moiety of the chimeric protein is localized in the periplasm, complementing the deletion.<sup>26</sup>

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