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# Determination of membrane protein stability via thermodynamic coupling of folding to thiol–disulfide interchange

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(RECEIVED March 12, 2003; FINAL REVISION May 5, 2003; ACCEPTED May 6, 2003)

## Abstract

Although progress has been made in understanding the thermodynamic stability of water-soluble proteins, our understanding of the folding of membrane proteins is at a relatively primitive level. A major obstacle to understanding the folding of membrane proteins is the discovery of systems in which the folding is in thermodynamic equilibrium, and the development of methods to quantitatively assess this equilibrium in micelles and bilayers. Here, we describe the application of disulfide cross-linking to quantitatively measure the thermodynamics of membrane protein association in detergent micelles. The method involves initiating disulfide cross-linking of a protein under reversible redox conditions in a thiol–disulfide buffer and quantitative assessment of the extent of cross-linking at equilibrium. The 19–46  $\alpha$ -helical transmembrane segment of the M2 protein from the influenza A virus was used as a model membrane protein system for this study. Previously it has been shown that transmembrane peptides from this protein specifically self-assemble into tetramers that retain the ability to bind to the drug amantadine. We used thiol–disulfide exchange to quantitatively measure the tetramerization equilibrium of this transmembrane protein in dodecylphosphocholine (DPC) detergent micelles. The association constants obtained agree remarkably well with those derived from analytical ultracentrifugation studies. The experimental method established herein should provide a broadly applicable tool for thermodynamic studies of folding, oligomerization and protein–protein interactions of membrane proteins.

**Keywords:** Membrane proteins; thiol–disulfide interchange; thermodynamics; folding

**Supplemental material:** See [www.proteinscience.org](http://www.proteinscience.org)

The folding of  $\alpha$ -helical membrane proteins has been proposed to occur in two energetically well-separated steps involving (1) insertion and formation of transmembrane helices and (2) their association to give the fully folded structure (Popot and Engelman 1990, 2000). Although the first stage has been extensively studied (White and Wimley 1999), the understanding of how transmembrane helices as-

sociate into a native folded protein in a membrane environment has been limited by a lack of quantitative thermodynamic data. In contrast to water-soluble proteins, thermodynamic studies of membrane protein folding have proven to be extremely challenging, and complicated by the difficulty of finding conditions for reversible folding. Despite considerable efforts in this area, the number of reversible folding systems developed for thermodynamic studies of membrane proteins is quite limited (Chen and Gouaux 1999; Fleming and Engelman 2001; Gratkowski et al. 2001; Zhou et al. 2001). The effects of mutations on the thermodynamics of transmembrane helix association has been intensively investigated for small bundle proteins such as the glycoporphin dimer (Lemmon et al. 1992; Fisher et al. 1999),

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Article and publication are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.0378603>.

and the first quantitative measurements of the thermodynamics of folding of functional transmembrane bundles with a larger number of helices have only recently been reported (Howard et al. 2002). Several experimental approaches including Förster resonance energy transfer (FRET; Adair and Engelman 1994; Fisher et al. 1999; Choma et al. 2000) and equilibrium analytical ultracentrifugation (Fleming et al. 1997; Gratkowski et al. 2001; Howard et al. 2002) have been employed to measure the energetics of membrane protein oligomerization. However, the bulk of thermodynamic data comes from studies on detergent micelle solutions, which do not fully mimic the native membrane environment. Consequently, there is considerable interest in finding methods for thermodynamic measurements that can be used in both lipid bilayers as well as micelles.

In this study we explore the possibility of using thiol–disulfide exchange reactions to quantitatively measure the thermodynamics of transmembrane helix association in detergent micelles. Thiol–disulfide exchange reactions are important in a variety of biological systems, and have been widely used in studies of protein folding, structure, and stability (Baldwin 1975; O’Shea et al. 1989; Creighton 1992; Gilbert 1994; Wedemeyer et al. 2000; Cochran et al. 2001; Blandl et al. 2003; Russell et al. 2003). This method has also been used extensively to examine the coupled association/folding interactions of coiled coil peptides (Oakley and Kim 1997, 1998; McClain et al. 2001a,b). Further, in an elegant series of articles, Regen et al. have used this method to examine the preferential association of lipids in bilayers (Krisovitch and Regen 1991, 1992; Davidson and Regen 1997; Regen 2002; Sugahara et al. 2002). However, to date, this method does not appear to have been used to measure the thermodynamics of transmembrane helix association in membrane-like environments. The principle of measuring a reversible association reaction by disulfide cross-linking involves approaching the equilibrium from both directions using a redox couple, quenching of the reaction by lowering the pH, separation, and quantitation of the products. In this article we use HPLC to quantify the products; gel electrophoresis could also be applied for this purpose.

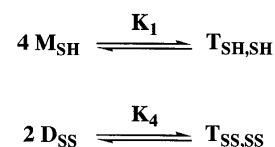
The 19–46 transmembrane segment of the M2 protein from the influenza A virus was employed as a model system to investigate the application of disulfide bond cross-linking to measure the energetics of peptide association in detergent micelles and lipid bilayers. M2 is a tetrameric integral membrane protein with a single transmembrane helix that functions as a proton channel (Pinto et al. 1992). CD and solid-state NMR studies showed that the transmembrane segment of M2 adopts an  $\alpha$ -helical structure in lipid vesicles (Duff et al. 1992; Kovacs and Cross 1997). A synthetic 25-residue peptide corresponding to the predicted transmembrane section of M2 (M2TM 22–46) has been shown to form  $\alpha$ -he-

lical secondary structure (Duff et al. 1992; Kovacs and Cross 1997), possess ion channel activity in planar bilayers (Duff and Ashley 1992; Tosteson et al. 1994) and exist in detergent micelles in a cooperative reversible monomer–tetramer equilibrium dependent on the pH and peptide-to-detergent ratio (Kochendoerfer et al. 1999; Salom et al. 2000). Thus, it is believed that the biological activity of the full-length protein resides in the transmembrane segment, which spans the membrane. Although there is as yet no high-resolution structure of the M2 transmembrane helical tetramer, the association of M2TM has been experimentally and computationally investigated in a variety of membrane-mimicking environments (Kovacs and Cross 1997; Pinto et al. 1997; Sanson et al. 1997; Bauer et al. 1999; Forrest et al. 2000; Kovacs et al. 2000; Song et al. 2000; Torres et al. 2000; Wang et al. 2001; Tian et al. 2002). The full-length protein, M2, contains three cysteine residues: cysteines 17 and 19, which form intermolecular disulfide bonds in the extracellular domain and cysteine 50 at the cytoplasmic domain, which is acylated (Holsinger and Lamb 1991; Sugrue and Hay 1991; Veit et al. 1991). Site-specific mutagenesis studies demonstrated that even though Cys 17 and 19 stabilize the tetramer through formation of disulfide bonds (Holsinger and Lamb 1991), their presence is not required for the function of the protein (Castrucci et al. 1997). Therefore, the 19–46 transmembrane fragment with the native Cys<sup>19</sup> (M2TM<sub>19–46</sub>: CNDSSDPLVVAASIIGILH LILWILDRL) at the N-terminus appears to be a favorable choice for the thermodynamic study of its tetramerization in membrane-like environments by disulfide cross-linking studies.

## Results

### Analytical ultracentrifugation

The success of the reversible thiol–disulfide exchange method developed in this work is dependent on the premise that the tetrameric form of the peptide should form disulfides more readily than the monomeric form, thereby allowing one to mathematically extract the dissociation constant for tetramerization interaction. Conversely, if this is correct the disulfide-bonded dimer (D<sub>SS</sub>) should form disulfide-bonded tetramers (T<sub>SS,SS</sub>) at concentrations significantly lower than those required to form fully reduced tetramers (T<sub>SH,SH</sub>) from the corresponding reduced monomers (M<sub>SH</sub>) (Scheme 1). We used analytical ultracentrifugation to test this possibility.



Scheme 1

The equilibrium association of M2TM<sub>19–46</sub> peptide in both reduced and oxidized states was determined by sedimentation equilibrium in DPC micelles utilizing methods analogous to those used to study other transmembrane fragments of M2 (Kochendoerfer et al. 1999; Salom et al. 2000; Howard et al. 2002). The monomeric and dimeric (oxidized) peptides were incorporated into DPC micelles at a DPC concentration (15 mM) higher than its critical micelle concentration (1 mM; Kochendoerfer et al. 1999). To eliminate the contribution of the DPC micelles to the peptide's sedimentation, the density of the solvent was adjusted with D<sub>2</sub>O to match the density of the detergent (Tanford et al. 1974).

The M2TM<sub>19–46</sub> peptide is essentially fully insoluble in water, in both the reduced and oxidized states. Thus, the appropriate concentration variable for analyzing the sedimentation equilibrium curves is the activity of the peptide in the micellar phase, which can be approximated as the mole fraction (MF) of the peptide (peptide/detergent ratio). The equilibrium radial concentration profile for the reduced peptide is well described by a fully cooperative monomer-tetramer equilibrium with a  $K_d$  of  $2.0 \times 10^{-7}$  MF<sup>3</sup> for the tetrameric dissociation constant (see supplemental Fig. S1-curve a). This value is identical to that reported previously for 22–46 TM segment (Salom et al. 2000), and corresponds to a midpoint of 1:136 (peptide/detergent ratio). From the sedimentation data for the oxidized peptide (see supplemental Fig. S1-curve b), a dissociation constant of  $6.2 \times 10^{-5}$  MF was derived for the monomer-dimer dissociation constant, which corresponds to a midpoint of 1:16,000 (peptide/detergent ratio).

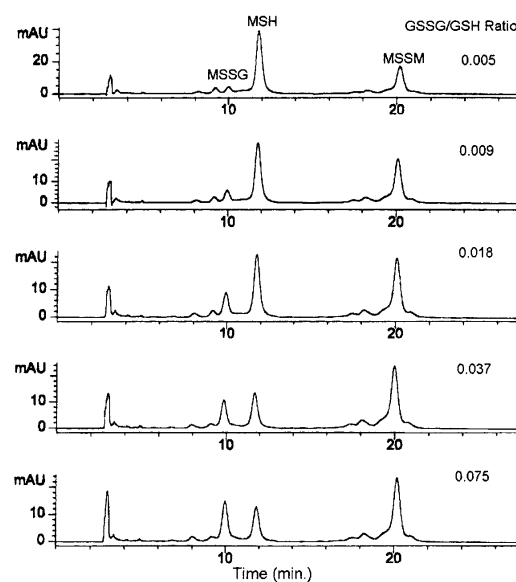
These two results indicate that the reduced and oxidized forms of M2TM<sub>19–46</sub> exist in a reversible equilibrium in DPC micelles, and that the oxidation greatly stabilizes the tetrameric (T<sub>SS,SS</sub>) form of the protein.

#### Equilibrium studies of M2TM<sub>19–46</sub> in detergent micelles

The above sedimentation studies indicate that the redox behavior of the M2TM peptide should depend on the peptide/detergent ratio, the peptide becoming more easy to oxidize as the concentration of peptide is increased. To test this prediction, the reduced form of M2TM<sub>19–46</sub> peptide was incorporated into DPC at various peptide-to-detergent molar ratios, in the presence of glutathione redox buffer containing known concentrations of oxidized (GSSG) and reduced (GSH) glutathione. The reaction was allowed to proceed to equilibrium, quenched by addition of HCl to effectively eliminate thiol exchange and oxidation, and the components present in the equilibrium mixture separated and quantified by analytical RP-HPLC.

Our first objective was to determine conditions under which the reaction was rapid and reversible. The formation or reduction of disulfide bonds by exchange with a linear or

cyclic disulfide-thiol reagent requires formation of a mixed disulfide intermediate (Creighton and Goldenberg 1984). Reagents such as glutathione promote the formation of stable mixed disulfides with protein thiols (Konishi et al. 1981; Creighton and Goldenberg 1984; Creighton 1986; Lin and Kim 1989). Utilization of too high concentrations of glutathione can lead to formation of mixed disulfides in high amounts, thus limiting the formation of the disulfide species of interest. Therefore, to determine the optimum conditions for disulfide cross-linking, different conditions were tested. The equilibration reactions were carried out by varying both the GSSG/GSH ratio and the total concentration of the two components. The oxidized and reduced glutathione forms were used in large excess over the peptide such that their concentrations at equilibrium can be considered unchanged while analyzing the equilibrium. Figure 1 shows a typical set of HPLC chromatograms of different equilibrium mixtures in a glutathione redox buffer containing 10.6 mM total glutathione and different GSSG/GSH ratios. Three major peaks are observed corresponding to the reduced (MSH) and oxidized (MSSM) peptides and mixed monodisulfide derivative (MSSG) of the peptide with glutathione. As Figure 1 indicates, the oxidized peptide accumulates under increasingly oxidizing conditions. However, an appreciable amount of mixed disulfide intermediate species forms and becomes kinetically trapped. Therefore, to reduce the extent of mixed disulfide species formation, we carried out the cross-linking measurements at a lower con-

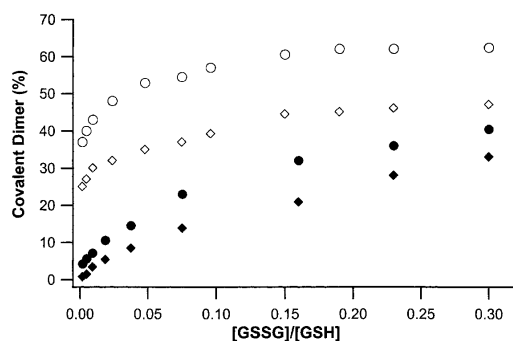


**Figure 1.** Analytical reverse-phase HPLC profiles of different equilibration mixtures in a glutathione redox buffer containing 10.6 mM total glutathione and different GSSG/GSH ratios. The peaks labeled MSH, MSSG, and MSSM correspond to the reduced, mixed disulfide of the protein with glutathione and oxidized species, respectively. Equilibrations were performed in 0.1 M Tris-HCl, 0.2 M KCl, 1 mM EDTA, pH 8.6. The peptide-to-detergent mole ratio was 1:200 in all samples.

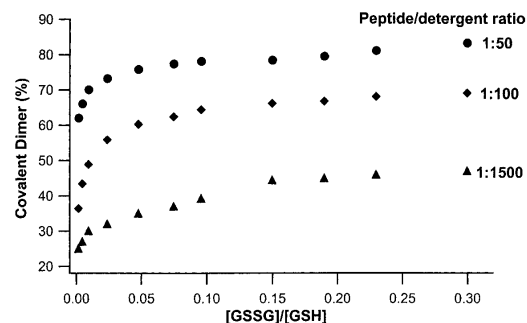
centration of glutathione (1.5 mM). Figure 2 compares the amount of dimer generated in the experiments conducted at 10.6 mM and 1.5 mM total glutathione for two different peptide-to-detergent mole ratios. It is evident from Figure 2 that the higher concentration of total glutathione leads to a greater tendency to form mixed disulfides between glutathione and protein, thus limiting the yield of dimer. By reducing the concentration of glutathione, the extent of dimer formation is significantly enhanced, and the process is readily reversible. When equilibration is carried out starting with the oxidized peptide (dimer) incorporated into DPC micelles, the two forms accumulate to the same extent as when the equilibrium is approached from the other direction by starting with the reduced peptide (see supplemental Fig. S2).

The sedimentation equilibrium study showed that the peptide exists in a reversible monomer–tetramer equilibrium, which depends on the amount of detergent, favoring monomers at low peptide-to-detergent ratios and tetramers at high peptide-to-detergent ratios. Therefore, one might anticipate that the extent of cross-linking be dependent on the peptide-to-detergent ratio; an enhanced ability to form disulfide-linked species is expected at high peptide-to-detergent ratios, which diminishes at low peptide-to-detergent ratios.

The percent of disulfide cross-linked product obtained from equilibration reactions carried out at different peptide-to-detergent mole ratios is plotted as a function of the GSSG/GSH ratio in Figure 3. Formation of oxidized species in Figure 3 is dependent on the peptide-to-detergent mole ratio in the expected qualitative manner. At high peptide-to-detergent mole ratios, the concentration of the protein in the micellar phase is increased and disulfide formation is enhanced, as reflected in the increase of the amount of dimer formed at equilibrium. At low peptide-to-detergent mole ratios the protein becomes predominantly monomeric



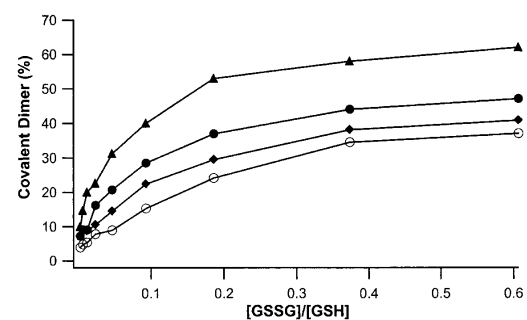
**Figure 2.** Comparison of the amount of covalent dimer generated in experiments conducted at 10.6 and 1.5 mM total glutathione for two peptide-to-DPC mole ratios. The following peptide-to-DPC mole ratios and concentrations of total glutathione were used: 1:1500, 10.6 mM (black diamonds), 1:1500, 1.5 mM (white diamonds), 1:200, 10.6 mM (black circles), 1:200, 1.5 mM (white circles).



**Figure 3.** Percent of covalent dimer as a function of the oxidizing potential expressed as [GSSG]/[GSH] for three different peptide-to-detergent mole ratios.

leading to a decrease in the extent of cross-linking. Thus, the percentage of total protein that exists as a dimer at equilibrium is diminished.

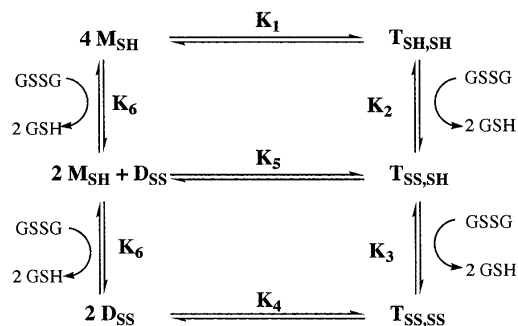
To confirm that the protein specifically associates into tetramers, we conducted the disulfide cross-linking experiments in the presence of amantadine. Previous studies showed that 22–46 TM segment of M2 forms amantadine-sensitive ion channels, and that binding of the drug favors tetramer formation (Salom et al. 2000). To investigate the effect of amantadine on the oligomerization of M2TM<sub>19–46</sub>, we conducted the disulfide bond formation experiments in the presence of amantadine. For this study, the peptide was incorporated into DPC at a low peptide to detergent ratio (1:1000); well below the midpoint for tetramer formation by the monomeric peptide. Different amounts of amantadine were added to the peptide/DPC samples and disulfide formation was initiated by adding GSSG and GSH in varying ratios. Figure 4 shows that addition of amantadine results in an enhancement of the cross-linking. The shift in the monomer–tetramer equilibrium induced by amantadine indicates that the protein specifically associates into tetramers, which bind amantadine.



**Figure 4.** Disulfide cross-linking in the presence of amantadine. White circles represent the data without amantadine. The ratio of peptide-to-DPC was 1:1000 in all samples. The final peptide-to-amantadine mole ratios were 1:2 (diamonds), 1:5 (black circles), 1:40 (triangles).

## Thermodynamic analysis

The model employed to describe the system and to account for various species present in the equilibration mixture is presented in Scheme 2.

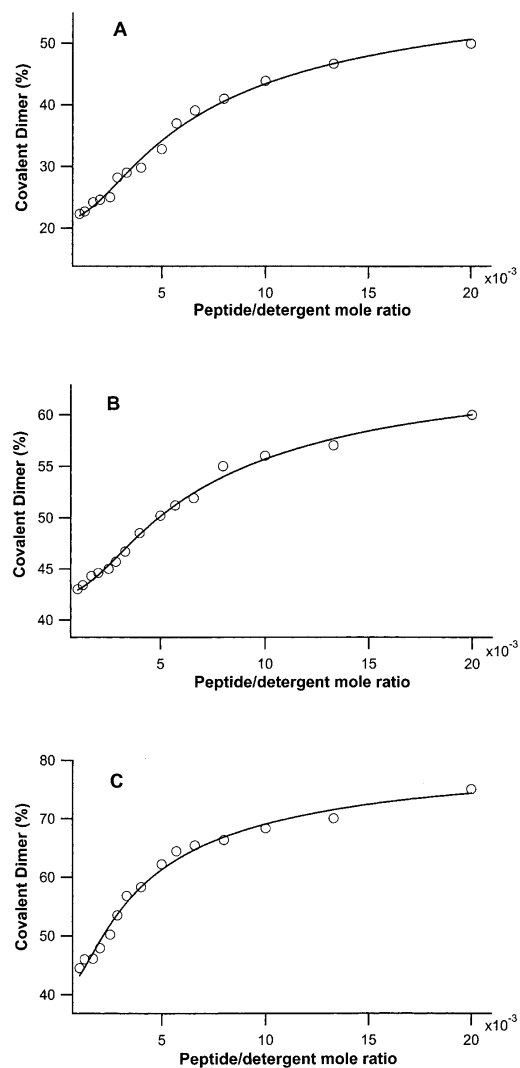


Scheme 2

$K_1$  represents the tetrameric dissociation constant,  $K_2$  is the equilibrium constant for the formation of the first disulfide bond,  $K_3$  is the equilibrium constant for the second disulfide bond formation, whereas  $K_4$  represents the dissociation constant for the dimer–tetramer equilibrium.  $M_{SH}$  is the reduced, monomeric peptide and  $T_{SH,SH}$  is the reduced, tetramer.  $T_{SS,SH}$ ,  $T_{SS,SS}$ , and  $D_{SS}$  are intermediate species and represent the one-disulfide tetramer, fully oxidized tetramer, and the oxidized peptide (disulfide-bonded dimer), respectively. The values of  $K_1$  and  $K_4$  were determined in the above section on analytical ultracentrifugation. These values will be compared with those derived from fitted parameters in the model presented in Scheme 2. The only constants that depend on the redox potential of the solution, expressed as  $[\text{GSSG}]/[\text{GSH}]^2$ , are  $K_2$ ,  $K_3$ , and  $K_6$ .

Because the redox buffer used in the above disulfide measurements contains very low starting GSSG to GSH ratios, that is, low concentrations of oxidized glutathione, quantitation of the data in the region corresponding to these low concentrations (Fig. 3) was prone to errors (because the equilibrium concentration of GSSG is low). Consequently, globally fitting the data in Figure 3 has proven to be problematic. To circumvent this problem, we conducted the equilibrations at sufficiently high GSSG to GSH ratios, where a more accurate measurement of the data is possible. The disulfide cross-linking reactions were carried out at three different fixed GSSG/GSH ratios (0.5, 0.2, and 0.1), and various peptide-to-detergent mole ratios.

Figure 5 illustrates the percentage of disulfide-bonded dimer generated from the three different experiments. The curves associated with the data in Figure 5 are fits obtained using the model in Scheme 1 (for details, see supplemental material). In fitting the data, we made the simplifying assumption that the intermediate state  $D_{SS}$  is populated at very



**Figure 5.** Calculated percent of dimer versus peptide-to-DPC mole ratio for three different  $[\text{GSSG}]/[\text{GSH}]$  ratios: 0.1 (A), 0.2 (B) and 0.5 (C). The lines are the best fits to the model described in the text.

low levels. This supposition is supported by the sedimentation equilibrium data, which indicate (a) a cooperative monomer–tetramer equilibrium and (b) a tight dimer to tetramer association.

Under these conditions, the total thermodynamic equilibrium is described by  $K_1$  through  $K_4$ , and it is not necessary to specify  $K_5$  and  $K_6$ . Further, to test the effect of  $K_5$  and  $K_6$  on the fit and the parameters obtained, the data were also fit to a model including  $K_5$  and  $K_6$ . No improvement in the fit was observed, nor were  $K_1$  and  $K_4$  affected, indicating that the most significant thermodynamic pathway is the one described by  $K_1$  through  $K_4$ . The values of the logarithm of any one of these parameters could be varied by up to 10% without significantly affecting the quality of fit of the curve to the data.

On the basis of the good fits obtained in Figure 5, it appears that the model illustrated in Scheme 1 describes the system very well. In Table 1 the dissociation constants derived from fitting the cross-linking data are reported as  $pK_{\text{diss}}$  values, and are compared to those obtained from analytical ultracentrifugation. As Table 1 shows, the values obtained for  $pK_1$  and  $pK_4$  from the cross-linking measurements are within experimental error and in consonance with those estimated from the equilibrium sedimentation study.  $pK_2$  and  $pK_3$  values are also in good agreement for measurements at all three GSSG/GSH ratios with the exception of  $pK_3$  at the lowest GSSG/GSH ratio employed (0.1). The equilibrium constant for the first disulfide bond formation ( $K_2$ ) is in the range of 3–7 M, whereas formation of the second disulfide bond has an equilibrium constant ( $K_3$ ) of ~1.5 M (for the latter, only the two  $K_3$  values corresponding to the highest GSSG/GSH ratios utilized (0.5 and 0.2) were considered—see Table 1). These constants are often considered as an effective concentration of the Cys residues, providing a quantitative measure of how readily each disulfide forms. The values obtained are very close to the range of values typically observed for Cys residues in native folded proteins (5–20 M, although values as high as  $1 \times 10^5$  M have been measured) (Lin and Kim 1989; Regan et al. 1994). Thus, these values are consistent with the Cys residues being presented from expected locations within short partially loops, which project from the same side of the bundle. The data also indicates that the formation of the first disulfide bond within the tetramer is more favorable than that of the second disulfide bond. Based on statistical considerations, one might indeed expect that the second disulfide bond will form with an equilibrium constant,  $K_3$ , of  $\sim(1/3)K_2$ , where 3 is a statistical factor that accounts for the fact that a given Cys has three potential partners in the fully reduced tetramer, but only one in the half-oxidized structure. (A given Cys residue has two potential partners on neighboring helices and one partner on the diagonally opposed helix in the tetramer.)

**Table 1.** Experimental values for  $pK_{\text{diss}}$  derived from cross-linking measurements in DPC micelles and analytical ultracentrifugation

[GSSG]/[GSH] ratio used in cross-linking studies	$pK_1$	$pK_4$	$pK_2$	$pK_3$
0.5	6.50	3.90	0.52	0.17
0.2	5.97	3.97	0.68	0.17
0.1	5.93	4.26	0.87	1.26
Analytical ultracentrifugation	6.68	4.21	—	—

The dissociation constants for all equilibria in Scheme 2 are reported as  $pK_{\text{diss}}$  ( $-\log_{10}$  of the dissociation constant expressed in peptide/detergent mole fraction (MF) units). They were determined from fitting the data in Figure 5 to the model described in the text. The values from analytical ultracentrifugation were obtained as indicated (see Results—Analytical ultracentrifugation section).

The good agreement between  $pK_1$  and  $pK_4$  values and those obtained from the analytical ultracentrifugation study strongly indicates that disulfide cross-linking can be used as a quantitative method to measure the association in detergent micelles.

## Discussion

The development of methods for reversibly folding membrane proteins in a two-state manner remains a considerable challenge for studies of membrane protein stability. In this article we describe a new experimental approach for investigating the thermodynamics of transmembrane helix–helix association in detergent solutions as well as in lipid bilayers. The 19–46 transmembrane segment of M2 protein from influenza A virus provides a good model for thermodynamic studies by this method. It has a moderate complexity (four-helix bundle), and it constitutes a representative example of the structure of a simple functional membrane protein. In addition, its reversible self-assembly in detergent micelles to give a single tetrameric form makes it a convenient system to study the association thermodynamics.

We have shown that M2TM<sub>19–46</sub> associates under oxidizing conditions to form disulfide-linked species in detergent micelles and this process has the expected dependence on the peptide-to-detergent mole ratio. A significant enhancement of the cross-linking is observed when performing the equilibrium measurements in the presence of amantadine. This effect is attributed to amantadine binding to the tetrameric form of the protein, which leads to a shift in the monomer–tetramer equilibrium towards the tetrameric species and thus, to an increase of the cross-linking. The main goal of this work was to demonstrate that this method can be applied to quantitatively assess the reversible peptide association in micelles. To this end, we compared the association constants ( $K_1$  and  $K_4$ ) with those derived from sedimentation equilibrium. There is a very good agreement of these values, demonstrating that the developed protocol can be used to measure the thermodynamics of helix–helix interactions in detergent micelles.

In conclusion, we have demonstrated that thiol–disulfide interchange provides a qualitative and quantitative method to explore the thermodynamics of transmembrane helix–helix association in detergent micelles. The ability to measure a reversible association reaction using this method in detergent micelles constitutes a valuable tool for the study of oligomerization and interactions of other membrane proteins, and may complement other measuring techniques. This method should also be quite applicable to the study of both monomeric and oligomeric membrane proteins. It is well known that the redox properties of water-soluble proteins depend markedly of the extent of folding (Anfinsen and Scheraga 1975; Creighton 1986, 1992; Creighton 1997; Wedemeyer et al. 2000), which could equally well be ap-

plied to study the folding of membrane proteins. Further, many membrane proteins can be split into two or more fragments that noncovalently associate to form a native structure (Popot and Engelman 1990, 2000). The introduction of appropriately placed Cys residues into such systems would provide a highly sensitive probe for folding.

## Materials and methods

### Peptide synthesis

M2TM<sub>19–46</sub> peptide (C-terminally amidated and N-terminally acetylated) was synthesized using an Applied Biosystems 430A peptide synthesizer. The peptide was synthesized on a Rink-amide resin with a substitution level of 0.78 mmole/g on a 0.25 mmole scale. Activation of the free amino acids was achieved with HBTU/HOBt/DIEA and 25% DMSO/75% NMP was used as solvent. The residues were double coupled and capped with acetic anhydride. Side chain deprotection and simultaneous cleavage from the resin was performed using a mixture of TFA/thioanisole/1,2-ethanedithiol/anisole (90:5:3:3 v/v) at room temperature, under nitrogen for 2 h. The crude peptide collected after precipitation with cold diethyl ether was dissolved in HPLC buffer B (6:3:1 2-propanol:acetonitrile:water) and lyophilized. The peptide was then purified by preparative reverse phase HPLC on a Waters HPLC using a Vydac C-4 preparative column using a linear gradient of buffer B (6:3:1 2-propanol:acetonitrile:water) containing 0.1% TFA and buffer A (0.1% TFA). Elution of the purified peptide occurred at approximately 80% buffer B. The homodimer was obtained by air oxidation at pH 8.8 followed by separation by RP-HPLC using a semipreparative Vydac C-4 column and the same HPLC buffers as described above. The identities of the purified monomeric peptide as well as that of the homodimer were confirmed by MALDI-TOF (PerSeptive Biosystems) mass spectrometry, and purity was assessed by analytical HPLC using an analytical C-4 column and a linear A/B gradient.

### Preparation of M2TM<sub>19–46</sub> detergent micelles

Samples were prepared by codissolving M2TM/trifluoroethanol (TFE) stock solutions with the appropriate amount of dodecylphosphocholine (DPC) (Avanti Polaris Lipids) stock solution in ethanol. The concentration of peptide (25  $\mu$ M) was kept constant while varying the DPC concentration to obtain the desired peptide-to-detergent molar ratios (typically between 1:50 and 1:1500). The solvent was evaporated under a stream of nitrogen; the protein/DPC film was kept overnight under high vacuum to remove all traces of solvent and then hydrated with 100 mM Tris.HCl, 0.2 M KCl, 1 mM EDTA pH 8.6 buffer. Incorporation of amantadine into the peptide/DPC samples was carried out as follows: the desired amount of drug was first added from a TFE stock solution to a glass vial and the solvent was evaporated under nitrogen. The peptide was incorporated into DPC at a peptide-to-detergent mole ratio of 1:1000; the samples were hydrated in buffer and added to the amantadine films under vigorous vortexing. The disulfide cross-linking reaction was initiated by adding oxidized (GSSG) and reduced (GSH) glutathione at varying ratios to the samples. The final molar ratios of peptide-to-DPC-to-amantadine were 1:1000:2, 1:1000:5, and 1:1000:40.

### Analytical ultracentrifugation

Sedimentation equilibrium experiments were conducted at 25°C on samples of reduced and oxidized (dimer) peptides incorporated into DPC micelles using a Beckman XL-I analytical ultracentrifuge. Experiments were carried out at a solvent density adjusted to equal that of DPC to eliminate the contribution of the buoyant molecular weight of the peptide-DPC complex (Tanford and Reynolds 1976; Furst 1997; Salom et al. 2000). The density matched buffer used was 0.1 M Tris-HCl, 0.2 M KCl, pH 8.6, and 40.4% D<sub>2</sub>O. For sedimentation of monomeric peptide, the density matched buffer also included 1 mM TCEP. Peptide samples were prepared by codissolving the desired amounts of monomeric or dimeric peptide with DPC, removing the solvent under nitrogen, keeping the samples under vacuum overnight, and then hydrating them with the density matched buffer. The molecular masses and partial specific volumes for monomer and dimer were calculated using the program Sedinterp (Laue et al. 1992). These calculated values were kept fixed when fitting the absorbance versus radius profiles to different equilibrium models. Data obtained were analyzed by nonlinear least-squares curve fitting of radial concentrations profiles by using the Marquardt-Lavengberg algorithm implemented in Igor Pro (Wavemetrics) as previously described (Kochendoerfer et al. 1999).

### Thiol–disulfide exchange equilibria with glutathione redox buffer

Thiol–disulfide exchange reactions of M2TM<sub>19–46</sub> incorporated into DPC micelles were carried out in a redox buffer containing reduced (GSH) and oxidized (GSSG) glutathione at varying ratios and total glutathione concentrations. The samples for equilibration measurements were prepared as follows: The peptide was first incorporated into DPC at various peptide-to-detergent mole ratios (typically between 1:50 and 1:1500). This was followed by the incubation of the peptide–DPC samples with a glutathione redox buffer in 0.1 M Tris-HCl, 0.2 M KCl, 1 mM EDTA pH 8.6. To test the optimum conditions for disulfide cross-linking, the equilibrations were performed at two total glutathione concentrations (10.6 and 1.5 mM), while varying the GSSG/GSH ratio. The ratios of GSSG to GSH used in the redox buffer were 0.002, 0.005, 0.0094, 0.0188, 0.0377, 0.0754, 0.16, 0.23, and 0.30 for a total glutathione concentration of 10.6 mM, and 0.0018, 0.0048, 0.0096, 0.024, 0.048, 0.075, 0.096, 0.15, 0.19, 0.23, and 0.30 for a 1.5 mM total thiol concentration. The reaction was carried out under a slow stream of nitrogen gas in screw-capped glass vials to prevent air oxidation. The solutions were allowed to equilibrate and the equilibration was monitored by HPLC on aliquots over 4 h. Generally, full equilibration was reached after 3 h; the equilibrium was established as indicated by no change in the HPLC peak area of all species with time. To check the reversibility, equilibration reactions were carried out in the same manner as described above, but starting with the dimer incorporated into DPC and increasing the reducing composition of the redox buffer. The concentration of oxidized and reduced glutathione in each stock solution was determined by UV measurements, using an absorption coefficient of 273 M<sup>-1</sup>cm<sup>-1</sup> at 280 nm (in the case of oxidized glutathione and by titration with Ellman's reagent (DTNB), measuring the absorbance at 412 nm and using an absorption coefficient of 13,600 M<sup>-1</sup>cm<sup>-1</sup> in the case of reduced glutathione (Regan et al. 1994). Protein concentration was determined spectrophotometrically by measuring the absorbance at 280 nm; the extinction coefficient used was 5600 M<sup>-1</sup>cm<sup>-1</sup>.

After equilibration, the samples were quenched by addition of HCl to a final concentration of ~0.12 M prior to HPLC analysis. The products of the thiol/disulfide exchange reaction were analyzed by RP-HPLC on an analytical C-4 column using a linear buffer A/buffer B gradient. The different peaks corresponding to the thiol-free peptide, mixed disulfide of peptide with glutathione and disulfide-bonded peptide were identified using MALDI-TOF mass spectrometry. Assignment of the reduced and oxidized HPLC peaks was also achieved by HPLC analysis of authentic samples of the oxidized and reduced peptides. A small amount of the reaction mixture was used to determine the total free thiol content at the end of reaction by Ellman test. Assuming that in the HPLC of the equilibrated mixtures, all species were quantitatively eluted and exhibited identical extinction coefficients at 220 nm, the amount of dimer was calculated from the integrated HPLC peak areas of the present species in the chromatograms, using the software supplied with the HPLC.

### Electronic supplemental material

The supplemental information contains details of the procedure utilized in curve-fitting the data in this article. In addition, AUC results and data showing the reversibility of the disulfide-bonding process in DPC micelles are provided.

### Acknowledgments

This work was supported by grant GM 56423 from NIH. We thank David Salom for helpful discussions.

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