Mapping the folding pathway of the transmembrane protein DsbB by protein engineering

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The four-helical transmembrane protein DsbB (disulfide bond reducing protein B) folds and unfolds reversibly in mixed anionic/non-ionic micelles, consisting of an unfolding intermediate I and a rate-limiting transition state (TS) between I and the denatured state D. Here, I describe the analysis of the folding behavior of 12 different alanine-scanning mutants of DsbB. For all mutants, TS is as compact as D and there is an accelerating increase in compaction as the protein proceeds to I and the native state. This unusual pattern of consolidation may reflect significant amounts of secondary structure in D, analogous to a classical folding intermediate. Unexpectedly, an increase in apolar surface area upon mutation is stabilizing whereas an increase in polar surface area is destabilizing. This effect is probably dominated by the effect of the mutations on the structure of the denatured state. I observe clear Hammond postulate behavior, in which a destabilization of I moves it closer to D. \( \phi \)-Value analysis indicates that in TS, a folding nucleus consisting of two to three residues with \( \phi \)-values of >0.5 forms at one end of the transmembrane helices, which expands to include residues closer to the middle of the protein in I. Thus, folding proceeds from a highly polarized starting point.

Keywords: folding kinetics/Hammond postulate behavior/mixed micelles/site-directed mutagenesis/solvent-accessible surface area

Introduction

In the protein engineering approach, individual side chains are systematically mutated, typically by alanine-scanning, and the effects on a given biological process such as protein folding or an enzymatic reaction, are determined (Fersht, 1987, 1999; Fersht et al., 1992). This approach allows us to determine the extent to which a given non-covalent interaction is formed at different stages of this progress. The approach has simply revolutionized our understanding of mechanisms in protein science. Its power lies in the underlying simplicity of the approach: it can be applied to any given process where the individual steps of the mechanism, and the effects of mutagenesis on this approach, can be monitored, no matter by which technique. In protein folding the approach is typically to combine equilibrium measurements (to determine stability differences between the native and denatured state) and kinetic measurements (to determine activation barriers and the free energy levels of transient intermediates), though kinetic measurements by themselves can be sufficient (Otzen et al., 1994; Izhaki et al., 1995; Otzen, 2003).

Protein engineering has long since proved its worth in the folding of water-soluble proteins, and there is now growing interest in applying this approach to a better understanding of the folding of membrane proteins. The challenge in this context is to identify conditions which allow reversible folding and unfolding according to the principle of microscopic reversibility, i.e. the rate at which a certain (solvent) condition is attained should be the same independent of starting conditions. This is not a trivial undertaking, since membrane proteins are very fastidious in their demand for a comfortable amphiphilic environment to maintain the native state, and once this state is lost it is often difficult to regain it (Booth et al., 2001). In addition, \( \alpha \)-helical membrane proteins are usually so hydrophobic, due to the presence of consecutive stretches of hydrophobic amino acids in the transmembrane helices, that chemical denaturants like urea and guanidinium chloride do not provide sufficient solubilizing power to denature this class of proteins and are also poor starting points for refolding studies, leading to high levels of aggregation and inactivation (Gorzelle et al., 1999). This contrasts with bacterial outer membrane proteins, which typically form \( \beta \)-barrel structures and can be refolded from the urea-denatured state into surfactants or phospholipid vesicles by simple dilution (Kleinschmidt and Tamm, 1996; Kleinschmidt et al., 1999), enabling protein engineering studies in which refolding and unfolding take place over a relatively narrow but high urea concentration interval in the presence of liposomes (Huysmans et al., 2010).

Although phospholipid vesicles are the closest equivalent to the biological environment of a membrane protein, they do not lend themselves to reversible unfolding experiments for \( \alpha \)-helical membrane proteins. Although a membrane protein embedded in such a vesicle may be unfolded by gradual addition of a denaturing surfactant such as sodium dodecyl sulfate (SDS), the phase diagrams for the mixing of vesicles and SDS are complicated, involve phase changes which give rise to enormous light scattering artifacts (le Maire et al., 2000) and are not reversible on the millisecond time scale required for proper membrane protein folding studies. The most straightforward solution is, instead, to use mixed surfactant micelles, which in its simplest version consists of two components, namely a non-ionic surfactant stabilizing the native state (typically dodecyl maltoside, DDM) and a denaturing surfactant stabilizing the denatured state (typically SDS). Starting with the pioneering work by Lau and Bowie (1997), it has been observed that membrane proteins unfold cooperatively when the degree of unfolding is plotted versus the mole fraction SDS. This can be modeled based on an empirical relationship.
between the mole fraction of SDS and the apparent free energy of unfolding of the membrane protein in question, equivalent to the much-loved linear relationships between free energies of unfolding and denaturant concentrations (Tanford, 1970). The use of the SDS mole fraction, rather than absolute concentrations of surfactant, as a measure of denaturing potency makes intuitive sense from the perspective that the mixed micelle, and thus the composition of non-ionic and anionic surfactant, is the environment experienced by the individual membrane protein. Provided the ratio of surfactant to protein is large enough, each membrane protein is effectively infinitely diluted and does not experience contributions from other membrane proteins in each micelle. This approach has been used by Bowie and co-workers to carry out protein engineering studies on the quantitative effect of mutations on the stability of bacteriorhodopsin (Faham et al., 2004; Yohannan et al., 2004; Joh et al., 2008). Booth et al. building on their pedigree of kinetic studies of the folding of bacteriorhodopsin into mixed lipid/detergent (1,2-dimyristoyl-sn-glycero-3-phosphocholine, DMPC)/3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, CHAPS) micelles from the SDS-denatured state (Booth et al., 1995, 1997; Riley et al., 1997; Allen et al., 2001), have recently carried their project to its logical conclusion by measuring both rate constants of refolding (from SDS into DMPC/CHAPS micelles) and unfolding (from DMPC/CHAPS into SDS) and analyzing the data as a function of SDS mole fraction (a chevron plot) is very lop-sided and reveals that the transition state for unfolding is much closer to the denatured state than to the native state. Nevertheless most of the side-chain interactions in helix B probed by mutations are fully formed in the transition state (Curnow and Booth, 2009), apart from two mutations near the cytoplasmic end of the protein. Intriguingly, this relative lack of structure may be linked to the required movements of this part of the helix in the protein’s photo-cycle.

Here I present a protein engineering analysis of the folding of 12 different mutants of the membrane protein DsbB (disulfide bond reducing protein B) from the inner membrane of *Escherichia coli*. This 176-residue protein is involved together with the periplasmic protein DsbA in the formation of disulfide bonds in the cytoplasm (Bardwell et al., 1993; Kadokura et al., 2003). The recent crystal structure of the DsbA:DsbB complex (Inaba et al., 2006; Inaba and Ito, 2008) confirms previous predictions of the presence of four transmembrane helices connected by long loops, most of whose structure is too flexible to be defined in the crystal structure. Further insight into loop structures has been provided by a recent solution NMR structure (Zhou et al., 2008). I have previously shown that the protein unfolds reversibly in mixed micelles of SDS and DDM (Otzen, 2003). Although DsbB reversibly binds quinone co-factors as part of its redox activity, this does not lend itself readily to spectroscopic studies to monitor formation and loss of native structure. Artifacts associated with the transfer of Trp to micelles with different levels of polarity mean that equilibrium measurements of the unfolding of DsbB are not feasible based on Trp fluorescence (Otzen, 2003). However, kinetic studies circumvent these problems by focusing on the conformational changes that occur subsequent to the transfer to an environment with altered micellar composition. The kinetic data are consistent with a folding model involving the denatured state D, a folding intermediate I and the native state N according to Scheme 1:

\[
D \xrightarrow{k_f} I \xrightarrow{k_u} N, \quad \text{Scheme 1}
\]

where \(k_f = [I]/[N]\) and \(k_f\) and \(k_u\) describe the rate constants of refolding and unfolding from D to I and vice versa. On the basis of this scheme, it is possible to calculate the stabilities of the three states and their relative levels of compaction (Otzen, 2003) as well as determine their thermodynamic properties (Sehgal and Otzen, 2006). I analyze data provided by the 12 mutants to describe the relationship between stability and structural changes, movements of the transition state and the structure of the transition state between D and I as well as that of I itself. These demonstrate the great usefulness of mixed micelles in determining the individual steps in the folding of membrane proteins, and open up new opportunities for more detailed analysis of membrane proteins.

### Materials and methods

#### Protein production and characterization

All mutants of DsbB were prepared, expressed and purified as described (Otzen, 2003). For simplicity, the proteins were characterized with their C-terminal His tails intact. Folding and unfolding kinetics were carried out in 20 mM sodium phosphate pH 8.0, 0.1 M NaCl, 5 mM DDM at 25°C in different concentrations of SDS and analyzed as described (Otzen, 2003). Briefly, the observed rate constant \(k_{obs}\) (either from folding or unfolding studies) were plotted against bulk SDS mole fraction \(x_{SDS} = [SDS]/([SDS]+[DDM])\) using the following equation derived from Scheme 1 (Otzen, 2003):

\[
\log k_{obs} = \log \left( \frac{10^{\log k_f} + 10^{\log k_u} + m_x x_{SDS}}{1 + 10^{\log k_f} - m_x x_{SDS}} \right),
\]

where \(m_x\) and \(m_f\) describe the linear dependences of \(k_f\) and \(k_u\) on \(x_{SDS}\). Note that there is no \(m_u\)-value associated with \(k_u\). As seen from the flat baseline at low \(x_{SDS}\)-values, the refolding rate constant is essentially constant with respect to \(x_{SDS}\), leading to an apparent \(m_f\)-value of 0. The significance of this is discussed in the Results section. Inclusion of an \(m_f\)-value in Equation (1) does not improve the quality of the fit.

For experiments under reducing conditions, DTT from a freshly prepared 1 M stock solution was added to 20 mM to all solutions. DsbB was incubated with DTT for at least 30 min before the refolding or unfolding experiment. In addition to the 12 mutants described in this study, we attempted to overexpress the following mutants: FA20, AG24, PA40, GA53, GA56, LA59, GA61, PA65, PA68, YA71, AG81, GA84 and YA153. However, these mutants gave rise to low expression levels and unsatisfactory kinetic signals and were not characterized further.

#### Analysis of folding kinetics

For the analysis, we plotted the log of the observed rate constant \(k_{obs}\) versus bulk SDS mole fraction \(x_{SDS}\). An alternative approach would be to use the micellar mole
fraction $\chi_{\text{mic}}$, in which we take into consideration the fact that SDS and DDM do not partition into micelles equally well, owing to their differences in critical micelle concentration. The composition of the mixed micelles can be calculated theoretically from the Clint equation (Clint, 1975) but this presupposes ideal mixing. By measuring the critical micelle concentration of different molar compositions of SDS and DDM, it is possible to calculate the actual micellar composition (Sehgal et al., 2005). The relationship between micellar and bulk micelle composition is complicated and for SDS and DDM under our buffer conditions follows approximately an empirical relationship given by the following (Sehgal et al., 2005):

$$
\chi_{\text{mic}}^{\text{SDS}} = 0.016 + 1.67 \times \chi_{\text{SDS}} - 3.38 \times \chi_{\text{SDS}}^2 + 2.66 \times \chi_{\text{SDS}}^3.
$$

For mixed micelles composed of other pairs of non-ionic and ionic surfactants, we have shown that the use of $\chi_{\text{mic}}$ rather than $\chi_{\text{SDS}}$ leads to linear correlations with stability parameters such as the melting temperature derived from thermal scans when studying the stability of the outer membrane protein AIDA in mixed micelles (Sehgal et al., 2005). In the case of the SDS–DDM pair, however, little difference was observed in those studies (Sehgal et al., 2005), suggesting that the $\chi_{\text{SDS}}$ parameter nicely represents the micellar environment experienced by the membrane protein. Consistent with this, I did not observe any improvement of the quality of the fits for DsbB folding kinetics by this approach, nor in the correlations between the different parameters discussed in the Results and Discussion section (data not shown). Given that the $\chi_{\text{SDS}}$ plots provide perfectly adequate linear regions that fit our folding equation (see below) and given that we already depend on numerous assumptions in the interpretation of the kinetic data in a mixed micelle system, it seems unnecessary to introduce an additional complication in this way. All subsequent analysis is therefore based on simple bulk mole fractions.

**Structural analysis**

The structure of DsbB from the crystal structure of the DsbA:DsbB complex (Inaba et al., 2006) (PDB file 2H17) was used. Polar and apolar surface areas were calculated using GETAREA (Fraczkiewicz and Braun, 1998) with the associated web site http://curie.utmb.edu/getarea.html. For all truncation mutations, the appropriate mutant was constructed in silico by deleting the appropriate atoms from the mutated side-chain in the pdb file and subsequently using this file to calculate surface areas of different atom types. Changes in solvent-accessible polar and apolar surface area in the native state ($\Delta A_{\text{p}}$ and $\Delta A_{\text{ap}}$, respectively) were determined by subtracting wild type surface areas from mutant surface areas. Following the approach of Bowie and co-workers (Faham et al., 2004), I also took into account changes in the SDS-denatured state as follows. Four pdb files for the four individual $\alpha$-helices in DsbB (residues 16–36, 46–65, 72–93, and 144–162, respectively) were constructed by deleting the regions outside these individual sequences, and the appropriate mutant versions were constructed as above. Surface areas were calculated with GETAREA and changes in apolar and polar surface areas ($\Delta A_{\text{p}}$ and $\Delta A_{\text{ap}}$, respectively) for mutations within these helices were calculated as for the native state. The overall change in polar and apolar surface area was for each mutation calculated using $\Delta A = \Delta A_N - \Delta A_D$. For the mutation PA40 (which is in a loop between helices 1 and 2), $\Delta A_{\text{p}}$ was calculated using a value of 90.5 and 63.6 Å² for Pro and Ala side chains in the extended conformation (Creamer et al., 1997).

**Results and discussion**

**General description of the mutants**

Twelve mutants in addition to DsbB wild type were produced and purified. Mutations were selected to sample the transmembrane region as representatively as possible. In addition to the 12 mutations successfully produced and characterized, an equal number of other mutations failed to express. Eleven of the 12 successful mutations involve truncation of side chains in the transmembrane part of the protein, except for a few Gly positions where Ala was introduced. The 12th mutation, PA40, involved a mutation in the loop between helices 1 and 2. These 12 mutations were all subjected to the unfolding–refolding experiments described for wild type DsbB (Otzen, 2003). Briefly, refolding experiments were carried out by mixing unfolded DsbB in 5 mM SDS with excess of DDM to produce mixed micelles with sufficiently low mole fractions of SDS to allow the protein to refold. Conversely, unfolding was initiated by mixing native DsbB in 5 mM DDM with excess SDS to allow unfolding to occur. These led in all cases to one major exponential decay phase. Refolding data also included a minor decay phase; however, the amplitude and rate constant for this phase varied in unpredictable ways, probably due to the small magnitude of the signal. Consequently, only the major phase was used for analysis, just as for wild type DsbB (Otzen, 2003).

The rate constant for the major decay phase was plotted versus the bulk mole fraction of SDS ($\chi_{\text{SDS}}$) (Fig. 1). For all 12 mutants, the data fitted satisfactorily to the folding scheme used to fit the folding of wild type DsbB, both under reducing and oxidizing conditions (data summarized in Table I). These fits provide values for the three rate constants $k_1$, $k_2$, and $K_1$ and their associated $m$-values, which describe how the logarithm of these values depend on $\chi_{\text{SDS}}$.

The kinetic plots are in all cases characterized by the following three divisions: first, a baseline folding region up to around 0.3 $\chi_{\text{SDS}}$ (oxidized DsbB) or 0.15 $\chi_{\text{SDS}}$ (reduced SDS), in which the refolding rate constant is essentially constant. In this region, according to our folding model, the dominant reaction is the folding of denatured DsbB directly to the native state without any significantly populated intermediate. This baseline region is unusual in that the rate constant does not change significantly with $\chi_{\text{SDS}}$, rather than declining in a logarithmic way as seen for other membrane proteins such as wild type bacteriorhodopsin (Curnow and Booth, 2007) and GlpG (D.E.O., unpublished observations) [Note, however, that most of the bacteriorhodopsin mutants in helix B examined in a protein engineering study led to the same horizontal base line region (Curnow and Booth, 2009)].

This baseline region is followed by a transition region spanning 0.2–0.3 (oxidized DsbB) or 0.1–0.2 (reduced DsbB) $\chi_{\text{SDS}}$, in which the rate constant increases by approximately two orders of magnitude. Here the observed rate constant is determined by the accumulation of the unfolding...
intermediate I, through which unfolding occurs according to our folding model. Thus, the higher the fraction of the protein in the I state rather than the native state N, the faster unfolding can occur. Finally, above ca. 0.5 x SDS (oxidized DsbB) or 0.35–0.4 x SDS (reduced SDS), there is a linear increase in the observed rate constant. For a few of the mutants, there is a certain scatter in this region, which might be interpreted to indicate additional transitions. However, there is no systematic trend to this scatter (which leads to a slight apparent rise for some mutants and a flattening out for others) and I attribute this to experimental uncertainty associated with the very high rate constants in this part of the plot and the relatively small signal changes. For simplicity, these aspects are therefore subsumed under the general linear fit. In this region, I is the dominant species from which unfolding occurs, i.e. I equilibrates with N within the dead time of the mixing. As seen from the data for log Ki and mi in Table I, the inflection leading to the linear region occurs at a value of xSDS where log Ki ≒ 0, i.e. I and N are equally populated at this point. The high value of mi (6–12 per mole fraction unit) means that within 0.1 mole fraction above this inflection point, I has risen to constitute around 80–95% of the ground state population.

Since the original publication of the data for wild type DsbB (Otzen, 2003), the subsequent study by Curnow and Booth (2007) demonstrated that unfolding kinetics of bacteriorhodopsin proceeded in a linear fashion and did not show a kink in the unfolding link. Thus, it seems well-founded to conclude that the inflection seen for DsbB around 0.35–0.5 x SDS is a genuine reflection of a change in the ground state for unfolding, rather than an intrinsic artifact of mixed micelles.

It is also to be expected that the reduced state is generally less stable than the oxidized state due to the loss of the two disulfide bonds stabilizing the loops of DsbB. This means that lower xSDS-values are required to unfold reduced compared with oxidized DsbB.

Significance of the kinetic m-values

In our analysis, the m-values describe the way in which the log of the kinetic parameters depend on xSDS. However, there is more to them than this simple mathematical relationship. For globular proteins denatured in chemical denaturants such as urea and guanidinium chloride, m-values are generally interpreted as the difference in burial between the different states (Tanford, 1970; Fersht, 1999). This is based on the consideration that denaturants are preferentially albeit weakly bound to the protein surface (Timasheff, 2002), and the greater the amount of accessible surface area, the greater the degree of binding. Thus more expanded states with more surface area are preferentially stabilized by chemical denaturants, and the m-values provide a quantitative measure of this preference, providing in essence a number for the relative change in surface area accompanying unfolding. This interpretation is further substantiated by the close similarity between denaturant-based m-values and an independently derived parameter, the specific heat capacity DCp (Schindler and Schmid, 1996; Otzen and Oliveberg, 2004).

It remains an open question whether the same reasoning can be applied to the unfolding of membrane proteins in mixed micelles. The simple relationships in chemical denaturants benefit from the very weak binding affinities, which makes the binding affinity very general and relatively insensitive to amino acid composition. In contrast, SDS as an ionic surfactant is able to bind with high affinity at low surfactant concentrations (Tanford, 1980; Renthal, 2006), typically mediated by a combination of strong complementary electrostatic interactions to basic side chains and burial of the alkyl chain in hydrophobic patches on the protein surface. This binding has been documented in most detail under conditions where SDS binds to the native state as a monomeric ligand without disrupting the overall protein structure (Yonath et al., 1977). However, similar considerations apply when SDS is present at the micellar concentrations required for denaturation, since micelle–protein
Table I. Summary of kinetic parameters for refolding and unfolding of DsbB mutants in mixed micelles of SDS and DDM

<table>
<thead>
<tr>
<th>Oxidizing Mutant</th>
<th>Log ( k_f ) (s(^{-1}))</th>
<th>Log ( k_u ) (s(^{-1}))</th>
<th>( m_u ) (M(^{-1}))</th>
<th>Log ( K_f )</th>
<th>( m_f ) (M(^{-1}))</th>
<th>Log ( K_{D-N} )</th>
<th>( m_{D-N} ) (M(^{-1}))</th>
<th>( \Delta G_{D-N} ) (kcal/mol)</th>
<th>( \Delta \Delta G_D ) (kcal/mol)</th>
<th>( \Delta \Delta G_I ) (kcal/mol)</th>
<th>( \Delta \Delta G_T ) (kcal/mol)</th>
<th>( \phi_I )</th>
<th>( \phi_T )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>-0.49 ± 0.11</td>
<td>0.1 ± 0.4</td>
<td>1.8 ± 0.5</td>
<td>-3.6 ± 1.0</td>
<td>7.3 ± 2.7</td>
<td>3.1 ± 1.0</td>
<td>9.1 ± 2.7</td>
<td>4.2 ± 1.4</td>
<td>=0</td>
<td>=0</td>
<td>=0</td>
<td>=0</td>
<td>NA</td>
</tr>
<tr>
<td>AG19</td>
<td>-0.42 ± 0.07</td>
<td>0.39 ± 0.21</td>
<td>1.6 ± 0.3</td>
<td>-4.2 ± 0.7</td>
<td>9.2 ± 1.9</td>
<td>3.4 ± 0.7</td>
<td>10.8 ± 1.9</td>
<td>4.6 ± 1.0</td>
<td>-0.44</td>
<td>-0.10</td>
<td>0.42</td>
<td>0.32</td>
<td>0.22</td>
</tr>
<tr>
<td>EL26</td>
<td>-0.16 ± 0.03</td>
<td>0.25 ± 0.11</td>
<td>2.0 ± 0.1</td>
<td>-5.2 ± 0.7</td>
<td>12.0 ± 1.9</td>
<td>4.7 ± 0.7</td>
<td>14.0 ± 1.9</td>
<td>6.5 ± 1.0</td>
<td>-2.29</td>
<td>-0.45</td>
<td>0.23</td>
<td>-0.22</td>
<td>0.20</td>
</tr>
<tr>
<td>AG29</td>
<td>-0.27 ± 0.04</td>
<td>0.5 ± 0.5</td>
<td>1.2 ± 0.6</td>
<td>-4.2 ± 0.4</td>
<td>7.0 ± 0.91</td>
<td>3.4 ± 0.6</td>
<td>8.2 ± 1.1</td>
<td>4.6 ± 0.9</td>
<td>-0.45</td>
<td>-0.30</td>
<td>0.61</td>
<td>0.31</td>
<td>0.66</td>
</tr>
<tr>
<td>PA40</td>
<td>-0.23 ± 0.08</td>
<td>0.56 ± 0.5</td>
<td>1.3 ± 0.5</td>
<td>-3.2 ± 0.5</td>
<td>6.1 ± 1.3</td>
<td>2.3 ± 0.7</td>
<td>7.4 ± 1.4</td>
<td>3.2 ± 0.9</td>
<td>0.98</td>
<td>-0.35</td>
<td>0.68</td>
<td>0.32</td>
<td>-0.36</td>
</tr>
<tr>
<td>AG57</td>
<td>-0.84 ± 0.07</td>
<td>0.97 ± 0.20</td>
<td>0.8 ± 0.2</td>
<td>-4.3 ± 0.3</td>
<td>8.8 ± 0.80</td>
<td>2.5 ± 0.4</td>
<td>9.6 ± 0.8</td>
<td>3.4 ± 0.5</td>
<td>0.74</td>
<td>0.48</td>
<td>1.21</td>
<td>1.68</td>
<td>0.64</td>
</tr>
<tr>
<td>AG66</td>
<td>-1.33 ± 0.11</td>
<td>0.2 ± 0.8</td>
<td>1.8 ± 0.5</td>
<td>-3.6 ± 0.6</td>
<td>6.2 ± 1.2</td>
<td>2.1 ± 1.1</td>
<td>8.0 ± 1.4</td>
<td>2.8 ± 1.4</td>
<td>1.33</td>
<td>1.14</td>
<td>1.14</td>
<td>1.14</td>
<td>0.19</td>
</tr>
<tr>
<td>FA82</td>
<td>-0.48 ± 0.08</td>
<td>2.04 ± 0.19</td>
<td>-0.2 ± 0.3</td>
<td>-4.2 ± 0.5</td>
<td>5.7 ± 0.73</td>
<td>1.6 ± 0.5</td>
<td>5.6 ± 0.8</td>
<td>2.2 ± 0.7</td>
<td>1.93</td>
<td>-0.01</td>
<td>2.66</td>
<td>2.65</td>
<td>-0.01</td>
</tr>
<tr>
<td>RA93</td>
<td>-0.09 ± 0.05</td>
<td>1.0 ± 0.4</td>
<td>0.7 ± 0.3</td>
<td>-3.4 ± 0.3</td>
<td>6.3 ± 0.85</td>
<td>2.4 ± 0.5</td>
<td>6.9 ± 0.9</td>
<td>3.2 ± 0.7</td>
<td>0.97</td>
<td>-0.54</td>
<td>1.21</td>
<td>0.67</td>
<td>-0.56</td>
</tr>
<tr>
<td>YA89</td>
<td>-0.21 ± 0.05</td>
<td>0.37 ± 0.14</td>
<td>1.5 ± 0.2</td>
<td>-4.6 ± 0.8</td>
<td>10.8 ± 2.1</td>
<td>4.0 ± 0.8</td>
<td>12.2 ± 2.1</td>
<td>5.5 ± 1.0</td>
<td>-1.30</td>
<td>-0.38</td>
<td>0.39</td>
<td>0.01</td>
<td>0.29</td>
</tr>
<tr>
<td>GA148</td>
<td>-0.44 ± 0.04</td>
<td>-0.1 ± 0.16</td>
<td>2.0 ± 0.2</td>
<td>-3.3 ± 0.5</td>
<td>7.9 ± 1.4</td>
<td>3.0 ± 0.5</td>
<td>9.9 ± 1.5</td>
<td>4.0 ± 0.7</td>
<td>0.15</td>
<td>-0.07</td>
<td>-0.25</td>
<td>-0.32</td>
<td>b</td>
</tr>
<tr>
<td>AG152</td>
<td>-0.63 ± 0.09</td>
<td>0.1 ± 0.4</td>
<td>1.6 ± 0.4</td>
<td>-3.8 ± 0.8</td>
<td>8.0 ± 2.2</td>
<td>3.1 ± 0.9</td>
<td>9.6 ± 2.2</td>
<td>4.2 ± 1.2</td>
<td>-0.07</td>
<td>0.19</td>
<td>0.02</td>
<td>0.21</td>
<td>b</td>
</tr>
<tr>
<td>AG157</td>
<td>-0.41 ± 0.06</td>
<td>-0.24 ± 0.17</td>
<td>2.0 ± 0.2</td>
<td>-4.0 ± 0.3</td>
<td>10.0 ± 0.52</td>
<td>3.8 ± 0.4</td>
<td>11.9 ± 0.6</td>
<td>5.2 ± 0.5</td>
<td>-1.05</td>
<td>-0.11</td>
<td>-0.44</td>
<td>-0.55</td>
<td>0.10</td>
</tr>
</tbody>
</table>

The different parameters are calculated as follows: \( \log K_{D-N} = \log k_f - \log k_u - \log K_f; m_{D-N} = m_u + m_f; \Delta G_{D-N} = 1.36^* \log K_{D-N} - \Delta G_{D-N} = \Delta G_{\text{wildtype}} - \Delta G_{\text{mutant}}; \Delta \Delta G_D = \Delta \Delta G_T = -1.36^* (\log K_{D-N}^\text{mutant} - \log K_f^\text{wildtype}); \Delta G_{I} = \Delta \Delta G_D + \Delta \Delta G_T; \phi_I = \Delta G_I/\Delta G_{D-N} - \Delta G_{T}; \phi_T = \Delta G_I/\Delta G_{D-N} - \Delta G_{T}.

Errors on \( \phi \)-values are not indicated but are estimated to be around 30–50%.

*All experiments carried out in 20 mM sodium phosphate pH 8.0, 0.1 M NaCl, 5 mM DDM and varying concentrations of SDS at 25°C. Errors quoted are from the fits to the major observed rate constant.

bNot calculated because the low levels of destabilization (\( \Delta G_{D-N} \), \( \Delta G_I \), \( \Delta G_T \)) make a reliable estimate of \( \phi \)-values impossible.
interactions will also be stabilized by electrostatic complementarity in combination with hydrophobic surface burial. These strong interactions make the process very sensitive to changes in amino acid composition, so that simple substitutions of cationic side chains, for example, Ala can dramatically reduce the denaturing potency of SDS (Otzen et al., 1999). Conversely, negative charges reduce the impact of SDS, since its denaturing potency increases strongly in a pH-range where the negative side chains Asp and Glu become protonated and lose their negative charge (Otzen, 2002).

However, these considerations do not apply to DsbB. The only two charge-changing mutants (EL26 and RA83) show stability changes opposite to those one would expect from simple electrostatic considerations. Thus, EL26 (helix 1), which removes a negative charge and thus should promote binding of SDS, in actual fact shows the highest increase in stability compared with wild type DsbB, both in the oxidized and the reduced state (Table I). In contrast, RA83 (helix 3) is the most destabilized of all the mutants. This indicates that the impact of electrostatic mutations may in fact reflect intrinsic changes in stability of DsbB, and that m-values derived from mixed micelle kinetics may indeed be used to interpret structural changes. However, this does not rule out that differences in the way in which SDS and DDM bind to DsbB could affect the kinetics. In a thermodynamic analysis of the unfolding and refolding of DsbB at different temperatures, we observed a striking anomaly between the m-values (which indicated an expansion of the protein upon unfolding) and the heat capacity change (which indicated a lower amount of water bound in the unfolded state) (Sehgal and Otzen, 2006). We have interpreted this to mean that SDS binds more extensively than DDM to DsbB (e.g. in the large loops between the helices, which are presumably not hydrophobic enough to attract DDM), so that the protein is in fact shielded from water to a greater extent in the denatured state than the native state.

With these caveats in mind, let us now turn to the actual DsbB data. In all cases, we observe an essentially flat refolding baseline at low \( \chi_{\text{SDS}} \), indicating a very low m-value for this step. In our folding model, the refolding step represents the change from the SDS-denatured state D to the transition state (TS) between D and I. Thus, we must conclude, as for the wild type, that D and TS are very close to each other in terms of compaction for all the mutants we have probed. The step from TS to I is described by the unfolding rate constant \( k_u \), and its associated \( m_u \)-value. These values are in the order 1–2 per mole fraction (Table I). \( m_u \) in turn is 4–6-fold less than the \( m_l \)-value describing the transition from I to N. Thus, we see that as folding proceeds, there is a greater and greater degree of compaction, and the major consolidation occurs between the intermediate and the native state, meaning that the intermediate is in fact a rather loose and expanded state where side-chain interactions might not be expected to play a major role. This is a surprising observation which runs counter to the folding behavior of most water-soluble proteins passing through a kinetic intermediate. In these cases, the plot of log \( k_l \) versus denaturant concentration will show a roll-over at low denaturant concentrations, where the ground state from which unfolding occurs is the intermediate state, rather than the denatured state (Baldwin, 1996; Fersht, 1999). The roll-over (which sometimes reaches a near-horizontal line) reflects the fact that the intermediate is significantly more compact than the denatured state, so that once the intermediate state is reached, there is not a major increase in compaction before the transition state for unfolding.

What could be the reason for this difference? It should be borne in mind that the SDS-denatured state has almost native-like levels of secondary structure for DsbB (Otzen, 2003) due to the ability of SDS to stabilize the α-helical conformation (Jirgensons, 1967). This state is in some ways equivalent to the intermediate state assumed by globular proteins, which also in many cases has a high level of secondary structure albeit fluctuating tertiary structure due to a lack of stabilizing interactions between individual helices (Fersht, 1999). The SDS-denatured state is therefore significantly more compact than the chemically denatured state, which in many cases approximates the random coil (Tanford, 1968). Therefore the change in compaction for membrane proteins folding in mixed micelles occurs in a rather narrow interval compared with that of globular proteins. Given that the stabilizing interactions between the helices, which make up the final native state, are most likely only formed as the final step in the folding process, it is in fact to be expected that structural consolidations build up to an increasing extent throughout the folding reaction, so that the initial folding steps may represent relatively minor docking events which do not alter the extent of surfactant binding nearly as much as the later events, where surfactants may be ‘squeezed’ out of the near-native protein. Further details about these states are provided by the \( \phi \)-value analysis below.

**Correlations between stability and solvent-exposed surface area**

The kinetic parameters allow us to calculate the overall thermodynamic stability \( \Delta G_{\text{D–N}} \) of the different DsbB mutants in the absence of SDS (i.e. \( \chi_{\text{SDS}} = 0 \)) and thus the change in stability caused by the mutation \( \Delta G_{\text{D–N}}^{\text{mutation}} = \Delta G_{\text{D–N}}^{\text{wildtype}} - \Delta G_{\text{D–N}}^{\text{mutant}} \). The available crystal structure of DsbB in complex with DsBA (Inaba et al., 2006) in turn provides us with relatively reliable estimates of the change in polar and non-polar surface areas associated with each mutation in the native state. I simply truncate each mutated side-chain *in silico* and calculate the accessible polar and non-polar surface areas. However, given the high level of structure in the SDS-denatured state, we need to take into account how mutations will affect this state. This is done by following the approach of Bowie and co-workers (Faham et al., 2004), as detailed in the Materials and methods section. Figure 2 illustrates the correlation between changes in polar and non-polar surface area and \( \Delta G_{\text{D–N}}^{\text{mutation}} \).

Although the correlations are in general poor, there is one important trend: an increase in apolar surface area upon mutation is *stabilizing* whereas an increase in polar surface area is *destabilizing*. This is completely opposite to correlations observed for the stabilization of α-helices in water-soluble proteins, where increases in the solvent-accessible apolar surface area are destabilizing (Serrano et al., 1992), whereas increases in polar surface area are stabilizing, particularly around the caps of the helix (due to the ability to provide access to hydrogen-bonding water molecules and thus satisfy unmatched hydrogen-bonding donors/acceptors).
Fig. 2. Plots of changes in accessible (A–C) non-polar and (D–F) polar surface area versus $\Delta \Delta G^{\text{mutation}, \text{D-N}}$, the change in stability caused by different mutations. A negative value of $\Delta \Delta G^{\text{mutation}, \text{D-N}}$ indicates stabilization by the mutation. Three different sets of surface area data have been used: changes in surface area in the native state, in the denatured state (approximated by individual helices, cf. Materials and methods section) and the difference between the two. The straight lines indicate the best linear fits to each set of data (fits and correlation coefficients provided above each graph). All these data points represent mutations in the transmembrane region of DsbB. The single mutation in a loop in DsbB (PA40) has been excluded for this reason. Inclusion of this data point significantly reduces the quality of the linear fit. Error bars are calculated based on fits to data in Fig. 1 and Table I.
The best correlation is found for $\Delta A^D_{\text{apolar}}$, i.e. the change in the surface-accessible surface area in the denatured state (Fig. 2B), with $\Delta A^N_{\text{apolar}}$ showing a slightly worse correlation (Fig. 2A). The slope of the linear fit for $\Delta A^D_{\text{apolar}}$ is $16.7 \pm 5.7 \text{A}^2/\text{kcal/mol}$, corresponding to $60 \pm 20 \text{cal/mol/A}^2$, which is somewhat higher than—though equal within error to—the $38 \text{cal/mol/A}^2$ determined by Faham et al. (2004). The authors in this pioneering work point out that their value is similar to, if not slightly larger than, similar values obtained for water-soluble proteins, which can be taken to indicate that the hydrophobic effect remains an important stabilizing force in membrane protein folding, even though association occurs within a hydrophobic environment. This may reflect the fact that the packing of helices in the membrane environment is so highly complementary that it provides a great deal of van der Waals interaction energy which fortuitously is of the same magnitude as the hydrophobic effect. For DsbB; however, I observe what appears to be the opposite of the hydrophobic effect, given that it is more favorable to expose hydrophobic surface area (and thus reduce the amount of surface area buried in stabilizing van der Waals packing interactions). This phenomenon may simply reflect the fact that inappropriate values for changes in surface area are being used, given that $\Delta A^D_{\text{apolar}}$ and $\Delta A^N_{\text{apolar}}$ reflect differences in the surface areas of end states, rather than actual changes in surface area upon folding. Thus, it may be seen to have a stabilizing effect on the native state to increase the exposed apolar surface in the denatured state as this will ultimately allow more surface area to be buried upon unfolding to the native state. However, by the same line of thinking it should be stabilizing to minimize the exposed apolar state in the native state, in contrast to the observed correlation increased $\Delta A^N_{\text{apolar}}$ and increased stability. Any arguments that, e.g. DDM can bind to exposed apolar surface in the native state, and thus stabilize it, will have to explain why DDM cannot just as easily stabilize the denatured state in the same manner. This remains a perplexing observation that is difficult to explain.

Faham et al. used $\Delta A^N_{\text{apolar}} - \Delta A^D_{\text{apolar}}$ as a measure of the actual effect of the mutation on the change in apolar surface area accessibility upon folding. Since all the mutations plotted in Fig. 2 are from the transmembrane helices of DsbB, this value corresponds to changes in the availability of lipid binding sites and has been shown to correlate very significantly with changes in the mixed-micelle-based stability of bacteriorhodopsin (Creamer et al., 1997) and glycoporphin. Surprisingly, this value produces a very poor correlation for DsbB (Fig. 2C), indicating that at least for this protein, the difference between surface area changes in the native and denatured states is not a useful parameter to predict the effect of mutations on protein stability. This is rather difficult to rationalize, since we would expect the denatured state to show a significant amount of structure and indeed we observe a strong correlation between $\Delta A^D_{\text{apolar}}$ and $\Delta \Delta \Delta^\text{mutation}_{\text{apolar}}$. However, it may be that $\Delta A^N_{\text{apolar}}$ and $\Delta A^D_{\text{apolar}}$ have to be combined or weighted in a different manner to provide the most accurate description of the change in surface area. This highlights the importance of more detailed studies of the unfolded states of membrane proteins, since the nature of this state is key to understanding the driving forces behind membrane protein folding.

Hammond postulate behavior in membrane proteins

Given that the $m$-values provide information about the change in compaction associated with a given folding step, it is possible to use these values to define the position of different species on the reaction coordinate between the native and the denatured states (Tanford, 1970; Matouschek and Fersht, 1993; Matouschek et al., 1995; Fersht, 1999). This information is useful as it allows us to determine whether folding of the membrane protein follows Hammond postulate behavior. Hammond postulate behavior states that the closer two consecutive states in a given reaction sequence are in energy, the closer they will be in structure. In other words, if an intermediate is destabilized, its free energy will approach that of the denatured state, and so will the structure (Hammond, 1955). Thus, the change in the position of the intermediate on the reaction coordinate (its structure) will correlate with its stability, i.e. the rates of its formation and decay. Previous work on barnase (Matouschek and Fersht, 1993) and C12 (Matouschek et al., 1995) have shown clear evidence of such Hammond-type behavior, though in different ways. C12 folds as a single cooperative unit through one transition state and without any folding intermediate, and all mutations in the protein give rise to the same type of Hammond behavior. Mutations that lead to faster unfolding bring the transition state closer to the native state in terms of energy and also move its position on the reaction coordinate closer to the native state, while mutations that lead to slower refolding rates bring the transition state further from the denatured state in energy and also closer to the native state in structure (Matouschek et al., 1995). We have interpreted this to be a consequence of a relatively uniform degree of structure formation in the transition state (Matouschek et al., 1995). In contrast, different parts of barnase show different degrees of sensitivity to mutagenesis, i.e. different levels of transition state movement.

The reaction coordinates can be determined from $m$-values as follows: for the folding pathway $N \leftrightarrow I \leftrightarrow D$ described by DsbB, there will be two species on the pathway between D and N, namely the intermediate species I and the transition state between D and I (TS). $m_{D-N}$ describes the overall change in compaction between the starting point N and the endpoint D on the unfolding pathway involving and is equal to the sum $-m_I + m_D + m_N$. $m_I$ is preceded by a negative sign because it describes a movement in the opposite direction, i.e. from D to TS, while $m_D$ and $m_N$ both describe movement towards the denatured state (from N and TS, respectively). We can define N as having position 0 and D as having position 1. In practice $m_I = 0$, due to the absence of significant slope in the native state baseline region of the chevron plot, as discussed previously. This means that the TS is to all intents and purposes just as compact (or rather expanded) as the denatured state, and this level of compaction does not change with mutation (that does not mean to say that the transition state is identical to the denatured state in terms of structure at the level of side-chain residues, cf. the $\Delta \Delta$-value analysis below).

The only other species between D and N whose position can be determined is that of the intermediate state (the transition state between I and N cannot be characterized in this scheme, as we cannot determine the rates of its formation and decay). It follows that I’s position is described by the
\[
\beta_1 = \frac{m_I/m_{D-N}}{1 - (m_I/m_{D-N})} = 1 - \frac{m_I}{m_{D-N}}. \]

A strong correlation (correlation coefficient 0.8) is observed when we plot \( \beta_1 \) versus the stability of I \( (\log K_I) \) extrapolated to 0.5 mole fraction SDS (Fig. 3A). This is the most reliable region to determine \( \log K_I \), since this is the region where I becomes more stable than N so the curvature of the unfolding plot changes. The slope of the plot in Fig. 3A is positive, that is, the structure of I \( (\beta_1) \) approaches that of D as the stability of I increases relative to N. In this region, I is more stable than N, and D in turn is more stable than I. That is, an increase in the stability of I relative to N brings it closer in stability to D. If on the other hand we plot \( \beta_1 \) versus the stability of I \( (\log K_I) \) using the stability of I extrapolated to the absence of SDS, we only observe a weak correlation with a correlation coefficient of 0.29 (Fig. 3A). The poor correlation most likely reflects the long extrapolation from a region of the chevron plot with a very steep curvature, leading to a significant uncertainty in the estimation of \( \log K_I \). Despite this, we can still make an important conclusion: in this case the slope is negative, i.e. \( \beta_1 \) decreases (so I moves further away from D) as the stability of I increases. Note however that in this region where \( x_{SDS} = 0 \), I is less stable than N which is more stable than D. Thus by moving closer to N in energy, I in fact moves further away from D, consistent with the decrease in \( \beta_1 \)-values.

The data from the reduced state of DsbB lie interspersed with the data points for the oxidized state of DsbB (data not shown), i.e. there is no basis to conclude that the reduced and oxidized states follow different folding pathways from N to D.

An alternative way to analyze the data is to plot \( m_{u}/m_{D-N} \) versus \( \log k_u \). This parameter describes the activation energy for unfolding DsbB from I to the transition state between I and D. Thus, the faster the unfolding rate, the lower the activation energy and the closer I moves to TS in energy. Since the transition state is essentially identical in compaction...
(though not in overall structure), this provides a more direct measure of how the energy of I changes compared with the denatured state in comparison to the log $K_1$-values which provide energy relative to the native state (and whose energy level therefore changes compared with the denatured state). In fact the plots of $m_{D-N}$ versus log $k$, both demonstrate a very strong correlation (Fig. 3C and D). In both cases the plot is positive, that is, I approaches the reaction coordinate position of D as the activation barrier decreases in energy. This is because in both situations ($\chi_{SDS} = 0$ and $\chi_{SDS} = 0.5$), the activation barrier is significantly less stable than I, so that a decrease in activation energy in effect brings the two states closer to each other.

Unlike the situation for barnase (Matouschek and Fersht, 1993) and C12 (Matouschek et al., 1995), we do not have enough mutational data points to conclude whether different regions of the protein show different levels of sensitivity to mutationally induced changes in energy. However, it is safe to conclude that all four plots are consistent with Hammond postulate behavior in the membrane protein DsbB, further vindicating the use of mixed micelles to characterize the folding pathway of membrane proteins. This analysis underlines that folding of membrane proteins can to a large extent be treated in the same way as that of water-soluble proteins.

Curnow and Booth find anti-Hammond behavior for the folding/unfolding of bacteriorhodopsin in mixed SDS/DMPC/CHAPS micelles (Curnow and Booth, 2009), that is, the activation barrier for folding increases although the transition state apparently moves closer to the denatured state. They attribute this to effects on the denatured state of bacteriorhodopsin, so that the overall $m_{D-N}$-value is affected and this disproportionally affects the unfolding $m$-value, leading to an apparent movement of the transition state towards the denatured state. This does not appear to complicate the situation for DsbB, where anti-Hammond effects are not observed. Given that the denatured state is compact and has near-native levels of secondary structure for both proteins, it remains difficult to rationalize this difference. Further information may be obtained from a more detailed study of the SDS-denatured states of these two proteins.

**$\phi$-Value analysis of the folding of DsbB**

We now turn to a $\phi$-value analysis of the folding of DsbB, using the kinetic parameters derived from the chevron plots in Fig. 1 to calculate $\phi$-values for the transition state between D and I and for the intermediate state I. These values are calculated using the equations provided in Table I. Only in a few cases (AG152 and GA148) are the levels of destabilization induced by the mutation as low as to make reliable $\phi$-value estimates impossible. The kinetic values allow us to calculate two sets of $\phi$-values, namely $\phi_t$ (the $\phi$-value for the transition state between D and I) and $\phi_i$ (the $\phi$-value for I itself). There is a significant spread in the values of the $\phi$-values, but generally they increase as we go from the TS to I, consistent with an increased consolidation of structure as we approach the native state. The easiest way to display the distribution of $\phi$-values is to divide them into two classes, namely those with low $\phi$-values ($<0.2$) and those with high ($>0.5$); there are none in between. This provides a remarkably clear picture (Fig. 4A and B): in the transition state, the two mutations with high $\phi_t$-values (AG57 and AG62) are found in one end of the protein while the remaining mutations with low $\phi_t$-values are grouped in the other end. The exception is the mutation AG29, but this may be an artifact since a corresponding analysis using kinetic values at $\chi_{SDS} = 0.5$ reclassifies AG29 in the low-$\phi_t$ class while the mutation AG152 is 'upgraded' to high $\phi_t$, consistent with its position closer to residues 57 and 62. Gratifyingly, the picture for $\phi_t$ provides a simple extension of this situation: 57 and 62 remain in the 'high $\phi_t$' class which has now been expanded to include residues 82, 83 and 157. These residues are in the interface between the high and low $\phi$-value regions seen for $\phi_i$, suggesting that folding involves an almost wave-like extension of a folding nucleus from one end of the transmembrane helix bundle to the rest of the protein. The smaller number of mutations for which analysis under reducing conditions was possible (Table I) makes it difficult to identify any differences between the oxidized and reduced states on the folding pathway. However, note that essentially all mutations could be classified in the same color scheme in the reduced state (see legend to Fig. 4).

There is little analogous information from other $\alpha$-helical membrane proteins with which to compare. For bacteriorhodopsin, attention has focused mainly on helix B which has been shown to be largely formed in the transition state for folding (Curnow and Booth, 2009). Intriguingly, the outer membrane $\beta$-barrel protein PagP, the only other membrane protein to have had its folding behavior examined by $\phi$-value analysis, also folds through a highly polarized transition state (Huysmans et al., 2010), in which the overall shape of the $\beta$-barrel is formed but the N-terminal part of the barrel, which is close to the periplasmic side, is relatively unstructured. This part of the protein will subsequently form interactions with the N-terminal $\alpha$-helix that acts as a kind of clamp for the barrel, locking the barrel into the membrane. This has been suggested to drive a tilted vectorial insertion into the membrane so that the C-terminal part acts as an ice-breaker that leads the membrane transfer step. This would make sense from a biological point of view, given that outer membrane proteins fold into the outer membrane from the periplasmic space and obviously have to insert into this
membrane in one particular orientation. Whether analogous considerations can be made for α-helical membrane is an open question, given that helical membrane proteins most likely insert sideways into the membrane through the translocon apparatus (Hessa et al., 2005). Nevertheless, tilted migration through the membrane has also been proposed to occur for the α-helices in the voltage-sensor domain (Elinder et al., 2007) and the insertion of α-helical pore toxins (Bayley, 2009).

Assuming that the helices are released singly or in pairs from the translocon into the membrane, one would expect that from a vectorial point of view it would be sensible to allow folding to start from the parts which are released first. Nevertheless, the residues which form part of the folding nucleus in the transition state constitute residues 57 and 62 from helix 2 and possibly residue 152 from helix 4. This is consistent with a model in which essentially the whole of the protein has to be translocated into the membrane for folding to occur.

Note also that the unstructured part of DsbB in the transition state and intermediate states (the upper part of the protein in Fig. 4) is close to the periplasmic space. This suggests that the processes driving folding emerge from the part that is most deeply embedded in the lipid bilayer and for whom it may be easiest to start to develop tertiary structure due to their proximity in the membrane environment. In contrast, the exterior loops may be subsequently brought together by the assembly of the transmembrane helices which then act as an organizing scaffold. Further mutations in appropriate parts of DsbB may clarify this aspect.

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