Communication

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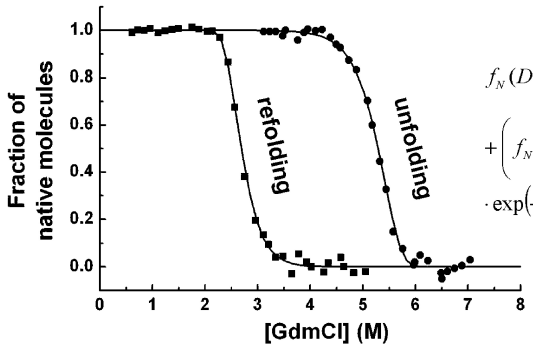

$$f_N(D,t) = \frac{k_F^0 \cdot e^{m_F \cdot D}}{k_F^0 \cdot e^{m_F \cdot D} + k_U^0 \cdot e^{m_U \cdot D}} + \left(f_N(0) - \frac{k_F^0 \cdot e^{m_F \cdot D}}{k_F^0 \cdot e^{m_F \cdot D} + k_U^0 \cdot e^{m_U \cdot D}} \right) \cdot \exp\left(- \left(k_F^0 \cdot e^{m_F \cdot D} + k_U^0 \cdot e^{m_U \cdot D} \right) \cdot t \right)$$

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Quantitative Analysis of Nonequilibrium, Denaturant-Dependent Protein Folding Transitions

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Many small, one-domain proteins show reversible, denaturant-induced unfolding/refolding transitions that are consistent with the two-state model of protein folding.[1] The model predicts that a protein preparation exists as a mixture of fully unfolded (U) and fully native (N) molecules.

$$\mathrm{U} \underset{k_U}{\overset{k_F}{\rightleftharpoons}} \mathrm{N} \tag{1}$$

The free energy of folding (ΔG^0) is then given by the expression

$$\Delta G^0 = -RT \ln \frac{k_F}{k_U} \tag{2}$$

where R is the gas constant, T is the absolute temperature, and k_F and k_U are the rate constants of folding and unfolding, respectively.

Denaturant-dependent unfolding/refolding transitions are most commonly used to determine protein stability. The native and the unfolded protein is diluted with buffers containing different denaturant concentrations, and the fraction of native and unfolded molecules after attainment of the equilibrium is measured spectroscopically.[1] The free energy of folding in the absence of denaturant, $\Delta G^0_{H_2O}$, is determined by linear extrapolation of the values of ΔG^0 to zero denaturant according to eq 3,[1]

$$\Delta G^0 = \Delta G^0_{H_2O} + m_{eq} D \tag{3}$$

where the equilibrium m-value, m_{eq}, corresponds to the cooperativity of folding and D is the denaturant concentration.

The values of $\Delta G^0_{H_2O}$ for small, one-domain proteins are in the range of -20 to -60 kJ mol^{-1}, and unfolding/refolding transitions generally attain equilibrium after incubation for several hours.[1] However, there is a considerable number of proteins that do not reach folding equilibrium within experimentally affordable time[2] and show unfolding transitions at high and refolding transitions at low denaturant concentrations. To our knowledge, a case of nonequilibrium behavior in denaturant-dependent protein folding with a comprehensive, quantitative evaluation of the unfolding and refolding transitions has not been reported so far.

Here we present a quantitative method for the analysis of nonequilibrium transitions in protein folding that is exclusively based on the dependence of the rate constants of unfolding and refolding on denaturant concentration. As a model protein, we used a self-complemented variant of the pilin domain of the *Escherichia coli* type 1 pilus adhesin, FimH$_P$.[3] FimH$_P$ has an incomplete, immunoglobulin-like fold that lacks the C-terminal G-strand (Figure 1a). In the pilus, FimH$_P$ interacts with an N-terminal extension of the neighboring subunit FimG that is inserted in an antiparallel orientation relative to the C-terminal G-strand. The self-complemented FimH$_P$ construct used in this study, FimH$_P$-DS$_G$ (139 residues; 14.1 kDa), is C-terminally extended by a β-turn sequence and the N-terminal extension of FimG, which enforces intramolecular self-complementation and donor strand insertion in the same orientation as predicted for the subunit−subunit interactions in the pilus[4] (Figure 1b).

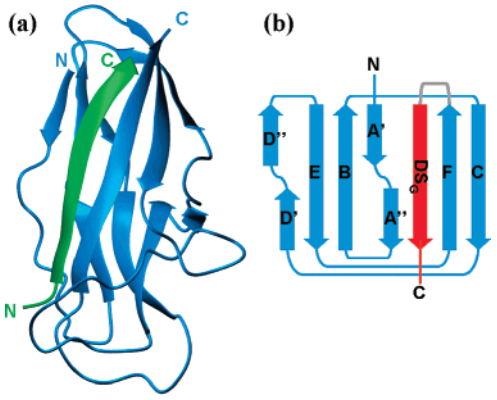

Figure 1. Donor strand complementation in FimH$_P$ (residues 158−279 of the type 1 pilus adhesin FimH[3]). (a) Ribbon diagram of the FimH$_P$ structure (blue) in complex with the chaperone FimC (pdb code 1QUN[4]). Residues 100−110 of FimC (green) are inserted parallel to the C-terminal F-strand of FimH$_P$. Residues 1−157 of FimH and residues 1−99 and 111−205 of FimC were omitted for clarity. (b) Topology diagram of intramolecular donor strand complementation in the construct FimH$_P$-DS$_G$. C-terminal elongation of FimH$_P$ (blue) by a β-turn sequence (gray) and the donor strand (residues 2−14) of the subunit FimG (red) enforces an antiparallel donor strand insertion relative to the F-strand of FimH$_P$.

Figure 2a shows that FimH$_P$-DS$_G$ exhibits extreme hysteresis-like behavior in its guanidinium chloride (GdmCl) dependent unfolding and refolding transitions at pH 7.0 and 25 °C. Albeit the unfolding and refolding transitions slowly move toward the equilibrium during several days, the equilibrium is far from being attained after 12 days. We performed a quantitative analysis of these data, assuming that (i) k_U and k_F are exponentially dependent on D (eq 4),[1] and (ii) that the folding equilibrium is attained according to a simple two-state equilibrium (eq 5), where k^0_F and k^0_U are the folding and unfolding rates at zero denaturant, m_F and m_U describe the dependence of ln(k_F) and ln(k_U) on D, and $f_N(t)$ is the fraction of N after incubation time t, with $f_N(0) = 1$ for the unfolding and $f_N(0) = 0$ for the refolding reaction.

$$\ln(k_F) = \ln(k^0_F) + m_F D$$
$$\ln(k_U) = \ln(k^0_U) + m_U D \tag{4}$$

$$f_N(t) = \frac{k_F}{k_F + k_U} + \left(f_N(0) - \frac{k_F}{k_F + k_U}\right) e^{(-(k_F + k_U)t)} \tag{5}$$

The combination of eqs 4 and 5 yields a general expression for the fraction of native molecules at any denaturant concentration and

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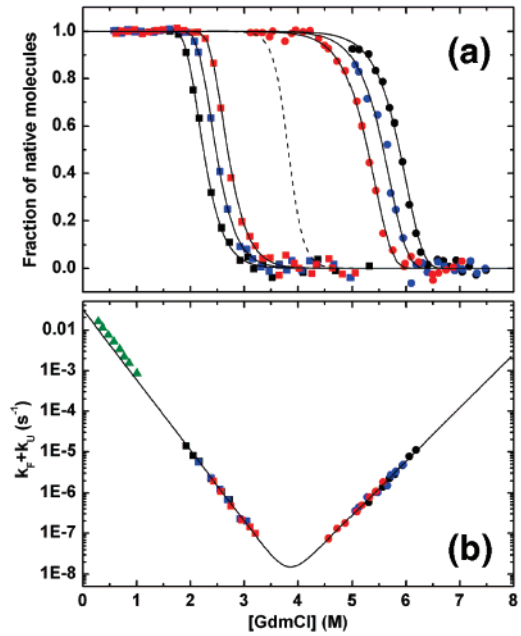

Figure 2. Nonequilibrium unfolding and refolding transitions of $FimH_P$-DS_G. (a) Normalized, GdmCl dependent unfolding (circles) and refolding (squares) transitions at pH 7.0 and 25 °C recorded after 2 days (black), 5 days (blue), and 12 days (red) of incubation. Solid lines represent the global fit according to eqs 6 and 7, the dashed line indicates the calculated equilibrium transition. (b) "Chevron plot" for the unfolding and refolding rates deduced from all data points in panel a for which the fraction of N is in the range of 0.1−0.9 (same symbols and color code as in panel a). The solid line shows the GdmCl dependence of the apparent rate constant of unfolding and refolding, which is the sum of k_F and k_U.[1] Refolding kinetics at low GdmCl concentrations (0.3−1.0 M) (green triangles; not included in the fit) confirm two-state folding within experimental error over the entire range of GdmCl concentration.

incubation time ($f_N(D,t)$) for the unfolding and the refolding reaction (eq 6).

$$f_N(D,t) = \frac{k_F^0 e^{m_F D}}{k_F^0 e^{m_F D} + k_U^0 e^{m_U D}} + \left(f_N(0) - \frac{k_F^0 e^{m_F D}}{k_F^0 e^{m_F D} + k_U^0 e^{m_U D}}\right) \exp(-(k_F^0 e^{m_F D} + k_U^0 e^{m_U D})t) \quad (6)$$

For analysis of the raw spectroscopic data recorded by circular dichroism (CD) spectroscopy, we assumed a linear dependence of the CD signal of N and U on D,[1] which yields eq 7 for the global analysis and normalization of the spectroscopic data.

$$S(D,t) = S_U^0 + n_U D + (S_F^0 + n_F D - S_U^0 - n_U D) f_N(D,t) \quad (7)$$

S_U^0 and S_F^0 are the spectroscopic signals of U and N at zero denaturant, and n_U and n_F are the dependencies of the signal of U and N on D, respectively.

The solid lines in Figure 2a correspond to the fit resulting from a global analysis of all transitions and normalization according to eq 7 and show that the data are in a very good agreement with the kinetic two-state analysis. We conclude that the hysteresis-like behavior of $FimH_P$-DS_G in unfolding and refolding transitions is a mere consequence of its extremely low rate constants of unfolding ($k_U^0 = 6.7 \pm 5.8\ 10^{-14}\ s^{-1}$; $m_U = 3.05 \pm 0.15\ M^{-1}$) and refolding ($k_F^0 = 3.2 \pm 0.5\ 10^{-2}\ s^{-1}$; $m_F = -4.00 \pm 0.08\ M^{-1}$). Figure 2b shows that folding of $FimH_P$-DS_G is more denaturant sensitive than unfolding, indicating that the solvent accessibility of the transition state is closer to that of N than U ($m_F/(m_U - m_F) = 0.57$).[1] The deduced half-life of folding ($t_{1/2} = 22$ s) at zero denaturant is similar to the even slower folding rate of the related type 1 pilus subunit FimG ($t_{1/2} = 160$ s).[5] Slow folding in the absence of cis-prolyl peptide bonds in the structure of $FimH_P$[4] and FimG[5] thus appears to be an intrinsic property of pilus subunits and explains why subunit folding is catalyzed by the type 1 pilus chaperone FimC in vivo[5].

We used the deduced values of k_F^0, k_U^0, m_F and m_U to calculate $\Delta G_{H_2O}^0$ of $FimH_P$-DS_G (eq 2) and the folding/unfolding transition at equilibrium (dashed line in Figure 2a). $FimH_P$-DS_G is a hyperstable protein with $\Delta G_{H_2O}^0 = -67 \pm 2.5$ kJ mol^{-1} and a transition midpoint at 3.83 M GdmCl. The cooperativity of folding (m_{eq}) of 17.5 ± 0.6 kJ mol^{-1} M^{-1} ($m_{eq} = RT(m_U - m_F)$) is in good agreement with the expected value for a 14.1 kDa protein.[1] We also used the kinetic parameters to predict the incubation time required to obtain experimentally indistinguishable unfolding and refolding transitions at 25 °C. Assuming an error of 2% for the CD signal at the transition midpoint, we calculated an incubation time of more than 9 years to attain 49% N in the refolding reaction and 51% N in the unfolding reaction at 3.83 M GdmCl.

In summary, we have demonstrated that apparent nonequilibrium behavior in the unfolding/refolding of monomeric $FimH_P$-DS_G can be quantitatively described with simple two-state kinetics and the exponential dependence of k_F and k_U on D. The analysis should be applicable to other proteins with nonequilibrium folding behavior and yields the values of $\Delta G_{H_2O}^0$, k_F^0, and k_U^0, and information on the solvent accessibility of the transition state relative to U and N. However, the absence of kinetic intermediates that slow the refolding reaction at low denaturant concentration[1] has to be confirmed independently by recording refolding kinetics. Equations 6 and 7 may also be applied to evaluate nonequilibrium unfolding transitions of oligomeric proteins and protein−ligand complexes for determination of their unfolding/dissociation rate in the absence of denaturant.

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Supporting Information Available: Amino acid sequence of $FimH_P$-DS_G and protocols for molecular cloning, protein expression and purification, and recording of folding transitions. This material is available free of charge via the Internet at http://pubs.acs.org.

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