Published in final edited form as:

Eur Biophys J. 2018 January ; 47(1): 89–94. doi:10.1007/s00249-017-1260-4.

Denaturant-specific effects on the structural energetics of a protein denatured ensemble

Mahdi Muhammad Moosa^{†,‡}, Asha Z. Goodman[†], Josephine C. Ferreon^{†,‡}, Chul Won Lee[§], Allan Chris M. Ferreon^{†,‡,*}, and Ashok A. Deniz^{†,*}

[†]Department of Integrative Structural and Computational Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA.

[§]Department of Chemistry, Chonnam National University, Gwangju 500-757, Republic of Korea.

[‡]Present Address: Department of Pharmacology & Chemical Biology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA.

Abstract

Protein thermodynamic stability is intricately linked to cellular function, and altered stability can lead to dysfunction and disease. The linear extrapolation model (LEM) is commonly used to obtain protein unfolding free energies ($\Delta G_{N \rightarrow D}^0$) by extrapolation of solvent denaturation data to zero denaturant concentration. However, for some proteins, different denaturants result in non-coincident LEM-derived $\Delta G_{N \rightarrow D}^0$ values, raising questions about the inherent assumption that the obtained $\Delta G_{N \rightarrow D}^0$ values are intrinsic to the protein. Here, we used single-molecule FRET measurements to better understand such discrepancies by directly probing changes in the dimensions of the protein G B1 domain (GB1), a well-studied protein folding model, upon urea and guanidine hydrochloride denaturation. A comparison of the results for the two denaturants suggests denaturant-specific structural energetics in the GB1 denatured ensemble, revealing a role of the denatured state in the variable thermodynamic behavior of proteins.

Keywords

single-molecule FRET; variable thermodynamics; two-state folding; solvent unfolding; protein folding

Proteins are the major workhorses among biomolecules in living systems, carrying out their functions through an intricate interplay between their structure and interactions. Globular proteins are normally required to adopt specific folded conformations to carry out their functions. The conversion of linear polypeptide chains into compact three-dimensional structures is driven by the inherent thermodynamic propensities of their amino acid sequences, modulated by the biomolecules' physicochemical environment and molecular

* deniz@scripps.edu, allan.ferreon@bcm.edu.

Associated Contents

Details of the experimental methods are provided in the Supporting Information.

interactions (Anfinsen 1973; England and Haran 2011). The role of conformational stability in the protein structure-function relationship is highlighted by the observed association of several diseases with mutations that cause protein stability alterations (Casadio et al. 2011). Consequently, the application of solvent denaturation to study the equilibrium thermodynamics and conformational energetics of folded proteins has been of considerable interest among protein chemists for more than six decades (Schellman 2002).

Protein conformational stability for a two-state folding protein exhibiting native (N) and denatured (D) states is defined thermodynamically in terms of the Gibbs free energy change of denaturation ($\Delta G_{N \rightarrow D}$). The experimental determination of $\Delta G_{N \rightarrow D}$ using solvent denaturation typically involves linear extrapolation of empirically observed free energy changes in the denaturation transition region to the limit of zero molar denaturant concentration (Greene and Pace 1974). The extrapolated value, $\Delta G_{N \rightarrow D}^o$, is usually assumed to be an inherent property of the protein under consideration, and interpreted as a denaturant-independent measurement of protein stability (Maxwell et al. 2005). Whereas this assumption of denaturant independence holds true for some proteins (Greene and Pace 1974; Santoro and Bolen 1988), the $\Delta G_{N \rightarrow D}^o$ values obtained from unfolding experiments using urea and guanidine hydrochloride (GdnHCl), the two most commonly used chemical denaturants, have been reported to be different for other protein systems (Bolen and Yang 2000; Ferreon and Bolen 2004; Monera et al. 1994; Pace 1986; Pace and Hermans 1975; Pace et al. 1990; Pace and Vanderburg 1979; Pfeil 1986).

To characterize why some proteins give denaturant-independent $\Delta G_{N \rightarrow D}^o$ values whereas others do not, Bolen and coworkers monitored proton inventories (a thermodynamic parameter) of several proteins upon GdnHCl denaturation (Bolen and Yang 2000), and classified proteins into three classes on the basis of their thermodynamic character (Ferreon and Bolen 2004) : a) proteins where individual states (N/D) are unaffected by the denaturant (fixed thermodynamic behavior); b) proteins where individual state(s) are perturbed outside the cooperative transition region (variable thermodynamic behavior outside the transition region); and c) proteins where individual state perturbation occurs simultaneously with the denaturation transition (variable thermodynamic behavior within the transition zone). The latter two categories of proteins can give rise to different LEM-derived $\Delta G_{N \rightarrow D}^o$ values for solvent denaturation experiments due to their denaturant-specific structural thermodynamics.

The immunoglobulin binding B1 domain of streptococcal protein G (GB1) is one of the most extensively studied model protein folding systems. The single-domain folded protein shows a two-state unfolding behavior in chemical denaturation with denaturant-specific $\Delta G_{N \rightarrow D}^o$ values (Ferreon and Bolen 2004). A previous study by Ferreon and Bolen characterized GB1 in terms of *within* state and *between* state effects of different denaturants on the unfolding equilibrium by monitoring unfolding-associated changes of a variety of spectral, dimensional and thermodynamic observables (Ferreon and Bolen 2004). The study revealed that GB1 exhibits *within* state effects on the N state ensemble at the pre-denaturation region upon GdnHCl-induced unfolding whereas urea-mediated unfolding

shows fixed thermodynamic behavior. However, effects of different chemical denaturants on the D state ensemble were not investigated. To further investigate GB1 variable thermodynamic character and to shed light on the denaturant-specific interactions of GB1 D ensemble, herein we directly probe changes in structures and dimensions of protein ensembles upon solvent-induced protein unfolding, employing a combination of single-molecule Förster resonance energy transfer (FRET) and ensemble far-UV CD spectroscopy.

The folded tertiary structures of globular proteins are stabilized by a combination of short- and long-range interactions involving the protein backbone and its side chains in physiological aqueous conditions (Dill et al. 2008). Chemical denaturants such as GdnHCl and urea disrupt these interactions either through direct protein interactions (Makhatadze and Privalov 1992) or indirect effects via the aqueous environment (Rezus and Bakker 2006), resulting in the destabilization of folded protein conformations. To monitor GB1 unfolding, we used far-UV CD spectroscopy to record changes in protein secondary structures (Greenfield and Fasman 1969; Greenfield 2006; Holzwarth and Doty 1965). Figure 1 shows the changes in mean residue ellipticity as functions of GdnHCl (a) and urea (b) concentrations. Clear cooperative transitions were observed for protein unfolding mediated by both denaturants. Each dataset was fitted to a two-state linear extrapolation model (LEM) of solvent denaturation using nonlinear least-squares (NLS) fitting as described by Santoro and Bolen (Santoro and Bolen 1988). Clearly, GdnHCl is more effective in denaturing GB1 compared to urea, with $C_{1/2}$ values of 2.17 ± 0.16 M and 6.2 ± 0.05 M respectively. This is consistent with previous observations of denaturant unfolding (Ferreon and Bolen 2004; Pace et al. 2008; Tanford 1968).

The valid application of LEM and determination of meaningful thermodynamic parameters requires proper handling of the pre- and post-transition baseline parameters. While the post-transition baseline of GdnHCl unfolding data and pre-transition baseline urea data were clearly evident, and could easily be determined programmatically by NLS fitting, determination of the other two baselines required manual intervention. For the pre-transition baseline of the GdnHCl unfolding data, the y -intercept was fixed at the observed MRE at 222 nm for 0 M denaturant. Determination of the post-transition baseline of the urea denaturation data required a further set of experiments because GB1 does not completely unfold at the highest urea concentration employed at $\text{pH } 5.0 \pm 0.05$ (Figure 1(b)). To obtain the post-transition baseline of urea-unfolded GB1, denaturant-mediated expansion was studied with alkaline pH-destabilized GB1 at $\text{pH } 11.0 \pm 0.1$ (Khare et al. 1997; O'Brien et al. 2011). The observed pattern of protein expansion (Figure 1(b)) is similar to previously observed denaturant-induced expansion of denatured and/or disordered protein ensembles (Baskakov and Bolen 1998; Ferreon et al. 2010; Yang et al. 2000). A linear post-transition baseline for the unfolded ensemble was obtained by fitting the urea unfolding data of GB1 at $\text{pH } 11.0 \pm 0.1$ in the range of 4.4 M – 9.35 M denaturant concentration to a linear function, and using the obtained slope and intercept parameters for the GB1 data at $\text{pH } 5.0 \pm 0.05$ (Figure 1(b)).

Green and Pace (Greene and Pace 1974) made the empirical observation that upon denaturation of proteins, the $\Delta G_{N \rightarrow D}$ values vary linearly in the transition region as a

function of denaturant concentration. A similar trend was observed for GB1 with both the chemical denaturants (Figure 1(c)). One of the fundamental assumptions of LEM is that this linear relationship between $\Delta G_{N \rightarrow D}^o$ and the denaturant can be extrapolated beyond the transition region to obtain denaturant-independent $\Delta G_{N \rightarrow D}^o$ values by extrapolating to the limit of zero molar denaturant concentration. However, extrapolation of our GdnHCl- and urea-induced GB1 unfolding data resulted in non-coincident $\Delta G_{N \rightarrow D}^o$ values (Figure 1(c)). Similar to the previously studied T2Q/I6A/T44A GB1 (Ferreon and Bolen 2004), the unfolding of D22C GB1 was consistent with a two-state transition and gave a higher extrapolated $\Delta G_{N \rightarrow D}^o$ value for urea-induced unfolding when compared with GdnHCl-mediated denaturation (Figure 1(c)).

Bolen and coworkers previously hypothesized that the observed discrepancies in the $\Delta G_{N \rightarrow D}^o$ values obtained from GdnHCl- and urea-induced unfolding experiments for a number of proteins are due to the *within* state effects in the N and/or D ensembles (Bolen and Yang 2000; Ferreon and Bolen 2004). While a detailed analysis of denaturant-mediated GB1 unfolding by Ferreon and Bolen (Ferreon and Bolen 2004) suggested variable thermodynamic nature of GB1 N ensemble, how different denaturants affect the D ensemble remained an open question. Here, to probe effects of the two chemical denaturants on the GB1 ensemble dimensions, we turned to single-molecule FRET experiments utilizing the distance dependence of the FRET efficiency (E_{FRET}) between donor and acceptor fluorophores. To monitor FRET at single-molecule resolution, we used a confocal detection setup, recording donor and acceptor signals as individual donor-acceptor-labeled GB1 molecules diffused through the sub-fL detection volume (Figure 2(a)). Single-molecule FRET measurements provide the ability to measure E_{FRET} (and hence distances) in the presence of labeling mixtures (e.g., molecules lacking fluorescent acceptor) and conformational subpopulations. For both denaturants, we observed a single non-zero FRET peak, which shifted as a function of denaturant concentration (Figure 2 (b) and (c)). Our observation is consistent with the urea-induced expansion of GB1 observed previously (Chung et al. 2009). The absence of multiple FRET peaks in the transition region suggests rapid inter-conversion between the N and D ensembles within the temporal resolution (500 μ s) of these experiments. For both denaturants, the single non-zero peak shifted towards lower E_{FRET} values with increasing denaturant concentration (Figure 2(b) and (c)) suggesting denaturant-mediated protein expansion.

Next, we plotted the non-zero smFRET peak positions against corresponding denaturant concentrations (Figure 3). The data revealed cooperative transitions and were analyzed using NLS fitting as described in the S.I. section, with fixed $\Delta G_{N \rightarrow D}^o$ and m -values obtained from ensemble experiments (Figure 1). For the urea unfolding data ($\text{pH } 5.0 \pm 0.05$), the slope of the post-transition baseline (dashed line; Figure 3 (b)) was fixed to the slope of the unfolded ensemble expansion data at $\text{pH } 11.0 \pm 0.1$ (dash-dot line; Figure 3(b)). All the fits had adjusted R^2 value of greater than or equal to 0.995. An independent fit of the single-molecule unfolding data also suggested similar extrapolated D ensemble dimensions. Similar to previously observed urea-mediated expansion of protein ensembles (Banerjee and

Deniz 2014; Ferreon et al. 2012; Huang et al. 2009; Schuler et al. 2002; Tischer and Auton 2013), pre- and post-transition baselines for both GdnHCl- and urea-mediated GB1 unfolding data had a negative slope suggesting denaturant-induced expansion in both N and D ensembles (Figure 3), albeit to a greater extent for the D ensemble.

From a polymer physics perspective, strong denaturants are good solvents that interact favorably with the protein backbone and individual peptide residues (Auton and Bolen 2005), resulting in expansion of proteins to more random-coil-like dimensions (Tanford 1968). Thus, it is expected that GdnHCl will be more efficient in expanding protein dimensions when compared to urea. This was indeed found to be the case as higher slopes were observed for pre- and post-transition baselines of the GdnHCl data when compared to those of the urea data in smFRET experiments (Figures 3). Interestingly however, the y -intercepts of the post-denaturation baselines of the two denaturation data reveal denaturant specific extrapolated unfolded ensemble dimensions (Figure 3), suggesting distinct structural energetics of the two denatured ensembles (as they exist in and are defined by the transition regions). Furthermore, the GdnHCl-induced unfolded ensemble (D_{GdnHCl}) showed a smaller dimension compared to the urea-mediated GB1 unfolded ensemble (D_{urea}) at the limit of 0 M denaturant concentration. While this appears counterintuitive on the first instance, it can be explained by the salt effect of GdnHCl (Smith and Scholtz 1996). Theoretical studies on the effects of salt on charged polymeric chains suggest that intra-chain electrostatic interactions are screened by salt ions, resulting in reduced persistence length and subsequent chain contraction (Dobrynin 2008). Thus, it is likely that the observed relatively compact denatured state upon GdnHCl-unfolding is due to the denaturant's salt effect on the D ensemble. Our observation also parallels previously observed non-linear effect of GdnHCl on intrinsically disordered (Muller-Spath et al. 2010) as well as denatured globular proteins (Tcherkasskaya et al. 2000).

A major proportion of studies on protein stabilities rely on the application of LEM to analyze and interpret data from protein solvent denaturation experiments. However, LEM-derived thermodynamic parameters obtained for a number of proteins show a dependence on both denaturant *nature* and *concentration*, raising questions about the validity and interpretation of the LEM-derived thermodynamic parameters for these proteins. An explanation of why some proteins exhibit denaturant-independent thermodynamic parameters upon linear extrapolation whereas others do not, lies in the presence of *within* state effects as well as their interplay with *between* state effects during protein denaturation. Here, we showed that GB1, a model protein for folding studies, shows denaturant-specific structural thermodynamics of its unfolded ensemble. Our observations suggest that similar to the previously reported variable thermodynamics of the GB1 N state ensemble (Ferreon and Bolen 2004), variable structural thermodynamics of D state ensembles can also contribute to the observed discrepancy in linearly extrapolated $\Delta G_{N \rightarrow D}^0$ values when using different chemical denaturants for several protein systems. Thus, one has to employ LEM cautiously taking into account of the possibility of variable thermodynamic character for either one or both of the protein (N and D) ensembles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We gratefully acknowledge support by grants MCB 1121959 (from the National Science Foundation to A.A.D.) and GM066833 (from NIGMS, National Institutes of Health to A.A.D.).

References

- Anfinsen CB (1973) Principles that govern the folding of protein chains. *Science* 181(4096):223–230 [PubMed: 4124164]
- Auton M, Bolen DW (2005) Predicting the energetics of osmolyte-induced protein folding/unfolding. *Proc. Natl. Acad. Sci. U. S. A* 102(42):15065–15068 [PubMed: 16214887]
- Banerjee PR, Deniz AA (2014) Shedding light on protein folding landscapes by single-molecule fluorescence. *Chem. Soc. Rev* 43(4):1172–1188 [PubMed: 24336839]
- Baskakov IV, Bolen DW (1998) Monitoring the sizes of denatured ensembles of staphylococcal nuclease proteins: Implications regarding *m* values, intermediates, and thermodynamics. *Biochemistry* 37(51):18010–18017 [PubMed: 9922169]
- Bolen DW, Yang M (2000) Effects of guanidine hydrochloride on the proton inventory of proteins: Implications on interpretations of protein stability. *Biochemistry* 39(49):15208–15216 [PubMed: 11106500]
- Casadio R, Vassura M, Tiwari S, Fariselli P, Luigi Martelli P (2011) Correlating disease-related mutations to their effect on protein stability: A large-scale analysis of the human proteome. *Hum. Mutat* 32(10):1161–1170 [PubMed: 21853506]
- Chung HS, Louis JM, Eaton WA (2009) Experimental determination of upper bound for transition path times in protein folding from single-molecule photon-by-photon trajectories. *Proc. Natl. Acad. Sci. U. S. A* 106(29):11837–11844 [PubMed: 19584244]
- Dill KA, Ozkan SB, Shell MS, Weikl TR (2008) The protein folding problem. *Annu. Rev. Biophys* 37:289–316 [PubMed: 18573083]
- Dobrynin AV (2008) Theory and simulations of charged polymers: From solution properties to polymeric nanomaterials. *Current Opinion in Colloid & Interface Science* 13(6):376–388
- England JL, Haran G (2011) Role of solvation effects in protein denaturation: From thermodynamics to single molecules and back. *Annu. Rev. Phys. Chem* 62(1):257–277 [PubMed: 21219136]
- Ferreon ACM, Bolen DW (2004) Thermodynamics of denaturant-induced unfolding of a protein that exhibits variable two-state denaturation. *Biochemistry* 43(42):13357–13369 [PubMed: 15491142]
- Ferreon ACM, Moosa MM, Gambin Y, Deniz AA (2012) Counteracting chemical chaperone effects on the single-molecule α -synuclein structural landscape. *Proc. Natl. Acad. Sci. U. S. A* 109(44):17826–17831 [PubMed: 22826265]
- Ferreon ACM, Moran CR, Gambin Y, Deniz AA (2010) Single-molecule fluorescence studies of intrinsically disordered proteins In: Walter NG (ed) *Methods Enzymol.* Academic Press, San Diego, pp 179–204
- Greene RF, Pace CN (1974) Urea and guanidine hydrochloride denaturation of ribonuclease, lysozyme, α -chymotrypsin, and β -lactoglobulin. *J. Biol. Chem* 249(17):5388–5393 [PubMed: 4416801]
- Greenfield N, Fasman GD (1969) Computed circular dichroism spectra for the evaluation of protein conformation. *Biochemistry* 8(10):4108–4116 [PubMed: 5346390]
- Greenfield NJ (2006) Using circular dichroism spectra to estimate protein secondary structure. *Nat. Protoc* 1(6):2876–2890 [PubMed: 17406547]
- Holzwarth G, Doty P (1965) The ultraviolet circular dichroism of polypeptides. *J. Am. Chem. Soc* 87:218–228 [PubMed: 14228459]

- Huang F, Ying L, Fersht AR (2009) Direct observation of barrier-limited folding of BBL by single-molecule fluorescence resonance energy transfer. *Proc. Natl. Acad. Sci. U. S. A* 106(38):16239–16244 [PubMed: 19805287]
- Khare D, Alexander P, Antosiewicz J, Bryan P, Gilson M, Orban J (1997) pKa measurements from nuclear magnetic resonance for the B1 and B2 immunoglobulin G-binding domains of protein G: Comparison with calculated values for nuclear magnetic resonance and X-ray structures. *Biochemistry* 36(12):3580–3589 [PubMed: 9132009]
- Makhatadze GI, Privalov PL (1992) Protein interactions with urea and guanidinium chloride: A calorimetric study. *J. Mol. Biol* 226(2):491–505 [PubMed: 1322462]
- Maxwell KL, Wildes D, Zarrine-Afsar A, De Los Rios MA, Brown AG, Friel CT, Hedberg L, Horng J-C, Bona D, Miller EJ, Vallée-Bélisle A, Main ERG, Bemporad F, Qiu L, Teilum K, Vu N-D, Edwards AM, Ruczinski I, Poulsen FM, Kragelund BB, Michnick SW, Chiti F, Bai Y, Hagen SJ, Serrano L, Oliveberg M, Raleigh DP, Wittung-Stafshede P, Radford SE, Jackson SE, Sosnick TR, Marqusee S, Davidson AR, Plaxco KW (2005) Protein folding: Defining a “standard” set of experimental conditions and a preliminary kinetic data set of two-state proteins. *Protein Sci.* 14(3): 602–616 [PubMed: 15689503]
- Monera OD, Kay CM, Hodges RS (1994) Protein denaturation with guanidine hydrochloride or urea provides a different estimate of stability depending on the contributions of electrostatic interactions. *Protein Sci.* 3(11):1984–1991 [PubMed: 7703845]
- Muller-Spath S, Soranno A, Hirschfeld V, Hofmann H, Ruegger S, Reymond L, Nettels D, Schuler B (2010) Charge interactions can dominate the dimensions of intrinsically disordered proteins. *Proc. Natl. Acad. Sci. U. S. A* 107(33):14609–14614 [PubMed: 20639465]
- O’Brien EP, Brooks BR, Thirumalai D (2011) Effects of pH on proteins: Predictions for Ensemble and Single-Molecule Pulling Experiments. *J. Am. Chem. Soc* 134(2):979–987 [PubMed: 22148729]
- Pace CN (1986) Determination and analysis of urea and guanidine hydrochloride denaturation curves In: Hirs CHW, Timasheff SN (eds) *Methods Enzymol.* Academic Press, San Diego, pp 266–280
- Pace CN, Grimsley GR, Scholtz JM (2008) Denaturation of Proteins by Urea and Guanidine Hydrochloride In: *Protein Science Encyclopedia.* Wiley-VCH Verlag GmbH & Co.
- Pace CN, Hermans J (1975) The stability of globular protein. *Crit. Rev. Biochem. Mol. Biol* 3(1):1–43
- Pace CN, Laurents DV, Thomson JA (1990) pH dependence of the urea and guanidine hydrochloride denaturation of ribonuclease A and ribonuclease T1. *Biochemistry* 29(10):2564–2572 [PubMed: 2110472]
- Pace CN, Vanderburg KE (1979) Determining globular protein stability: Guanidine hydrochloride denaturation of myoglobin. *Biochemistry* 18(2):288–292 [PubMed: 570408]
- Pfeil W (1986) Unfolding of Proteins In: Hinz H-J (ed) *Thermodynamic Data for Biochemistry and Biotechnology.* Springer-Verlag, Berlin, pp 349–376
- Rezus YL, Bakker HJ (2006) Effect of urea on the structural dynamics of water. *Proc. Natl. Acad. Sci. U. S. A* 103(49):18417–18420 [PubMed: 17116864]
- Santoro MM, Bolen DW (1988) Unfolding free energy changes determined by the linear extrapolation method. 1. Unfolding of phenylmethanesulfonyl α -chymotrypsin using different denaturants. *Biochemistry* 27(21):8063–8068 [PubMed: 3233195]
- Schellman JA (2002) Fifty years of solvent denaturation. *Biophys. Chem* 96(2–3):91–101 [PubMed: 12034431]
- Schuler B, Lipman EA, Eaton WA (2002) Probing the free-energy surface for protein folding with single-molecule fluorescence spectroscopy. *Nature* 419(6908):743–747 [PubMed: 12384704]
- Smith JS, Scholtz JM (1996) Guanidine hydrochloride unfolding of peptide helices: Separation of denaturant and salt effects. *Biochemistry* 35(22):7292–7297 [PubMed: 8679559]
- Tanford C (1968) Protein denaturation. *Adv. Protein Chem* 23:121–282 [PubMed: 4882248]
- Tcherkasskaya O, Knutson JR, Bowley SA, Frank MK, Gronenborn AM (2000) Nanosecond dynamics of the single tryptophan reveals multi-state equilibrium unfolding of protein GB1. *Biochemistry* 39(37):11216–11226 [PubMed: 10985767]
- Tischer A, Auton M (2013) Urea-temperature phase diagrams capture the thermodynamics of denatured state expansion that accompany protein unfolding. *Protein Sci.* 22(9):1147–1160 [PubMed: 23813497]

Yang M, Ferreon ACM, Bolen DW (2000) Structural thermodynamics of a random coil protein in guanidine hydrochloride. *Proteins Suppl* 4:44–49 [PubMed: 11013399]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1:

Protein G B1 domain (GB1) chemical denaturation using guanidine hydrochloride (GdnHCl) and urea yields denaturant-dependent unfolding free energies. Changes in protein secondary structure upon unfolding were monitored using far-UV CD spectroscopy. Presented in (a) are GB1 GdnHCl unfolding data at pH 5.0 ± 0.05 ($\square$). Shown in (b) are urea denaturation data at pH 5.0 ± 0.05 ($\circ$) and pH 11.0 ± 0.1 ($\diamond$). The solid curves in (a) and (b) represent the nonlinear least-squares (NLS) fits of the data to the linear extrapolation model (LEM) as described in the S.I. Materials and Methods section. Dotted and dashed lines represent folded and unfolded linear baselines, respectively. The LEM-derived $\Delta G_{N \rightarrow D}^0$ and m -value parameters are 2.41 ± 0.28 kcal-mol⁻¹ and 1.11 ± 0.09 kcal-mol⁻¹-M⁻¹ for the GdnHCl data and 3.10 ± 0.13 kcal-mol⁻¹ and 0.50 ± 0.02 kcal-mol⁻¹-M⁻¹ for the

urea data, respectively. (c) Experimentally-derived $\Delta G_{N \rightarrow D}$ values at the unfolding transition regions are plotted against denaturant concentrations.

Figure 2:

Denaturant-induced expansion of GB1 dimensions monitored using single-molecule Förster resonance energy transfer (FRET). (a) Representative bursts of donor and acceptor fluorescence observed from individual dye-labeled GB1 molecules. FRET efficiency (E_{FRET}) decreases with increasing denaturant concentrations, suggesting denaturant-induced expansion of protein dimension, a general phenomenon observed for both GdnHCl (b) and urea (c).

Figure 3:

Denaturant-induced expansion of GB1 native (N) and denatured (D) ensembles. Presented in (a) and (b) are E_{FRET} values plotted against GdnHCl ($\square$) and urea ($\circ$) concentrations, respectively, at pH 5.0 ± 0.05 . Data for urea-induced linear expansion of GB1 D ensemble at pH 11.0 ± 0.1 ($\diamond$) is also shown in (b). Dash-dot line represents fit of the protein expansion data to a linear function. Dotted and dashed lines indicate folded and unfolded baselines, respectively.