Thermal unfolding studies show the disease causing F508del mutation in CFTR thermodynamically destabilizes nucleotide-binding domain 1

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Abstract: Misfolding and degradation of CFTR is the cause of disease in patients with the most prevalent CFTR mutation, an in-frame deletion of phenylalanine (F508del), located in the first nucleotide-binding domain of human CFTR (hNBD1). Studies of (F508del)CFTR cellular folding suggest that both intra- and inter-domain folding is impaired. (F508del)CFTR is a temperature-sensitive mutant, that is, lowering growth temperature, improves both export, and plasma membrane residence times. Yet, paradoxically, F508del does not alter the fold of isolated hNBD1 nor did it seem to perturb its unfolding transition in previous isothermal chemical denaturation studies. We therefore studied the *in vitro* thermal unfolding of matched hNBD1 constructs $\pm$ F508del to shed light on the defective folding mechanism and the basis for the thermal instability of (F508del)CFTR. Using primarily differential scanning calorimetry (DSC) and circular dichroism, we show for all hNBD1 pairs studied, that F508del lowers the unfolding transition temperature (T_m) by 6–7°C and that unfolding occurs via a kinetically-controlled, irreversible transition in isolated monomers. A thermal unfolding mechanism is derived from nonlinear least squares fitting of comprehensive DSC data sets. All data are consistent with a simple three-state thermal unfolding mechanism for hNBD1 $\pm$ F508del: $N(\pm\text{MgATP}) \rightleftharpoons I_T(\pm\text{MgATP}) \rightarrow A_T \rightarrow (A_T)_n$. The equilibrium unfolding to intermediate, I_T , is followed by the rate-determining, irreversible formation of a partially folded, aggregation-prone, monomeric state, A_T , for which aggregation to $(A_T)_n$ and further unfolding occur with no detectable heat change. Fitted parameters indicate that F508del thermodynamically destabilizes the native state, N , and accelerates the formation of A_T .

Keywords: CFTR; cystic fibrosis; NBD1; (F508del)NBD1; calorimetry; thermal denaturation; protein stability; temperature correction

Additional Supporting Information can be found in the online version of this article.

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Introduction

Mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) are at the root of all CF disease and ~1500 mutations are known to contribute to disease.^{1,2} The most prevalent mutation is a deletion of F508 (F508del) within the first nucleotide binding domain (hNBD1) of CFTR, which is found in about 70% of all CF chromosomes. CFTR is a member of the ABC (ATP-binding cassette) transporter superfamily. It functions as a chloride ion channel in exocrine epithelial cells of the lungs, liver, skin, digestive and reproductive tracts. Structural and functional studies on CFTR indicate that channel gating involves complex and coordinated conformational changes that require changes in domain-domain interactions, such as those between NBD1 and NBD2, transmembrane helices, the NBDs and cytosolic loops that are contiguous with the transmembrane domains, and interactions of a regulatory (R) domain only found in CFTR. Phosphorylation of the R domain by protein kinase A activates CFTR (reviewed in Ref. 3).^{4,5}

Newly synthesized (F508del)CFTR accumulates in the endoplasmic reticulum (ER) and is eventually degraded by the ER-associated degradation pathway. At physiological temperature, none is processed normally or trafficked to the plasma membrane.^{3,6,7} This processing defect arises from the misfolding and/or partial folding of (F508del)CFTR, which triggers its degradation. The intracellular biosynthesis and folding pathway for CFTR is a complex process that involves both cotranslational folding of some domains, and post-translational folding and association of others.^{8–11}

Numerous *in vivo* studies of CFTR intracellular biosynthesis suggest that NBD1 folds relatively early and is only modestly perturbed in (F508del)CFTR. These and other *in vivo* studies,^{2,8–10,12} as well as *in vitro* folding of isolated hNBD1 constructs¹³ point to a crucial role for F508del in the disruption of post-translational, inter-domain assembly and folding of (F508del)CFTR. Because the F508 deletion produces only local conformational changes on the surface of the crystal structure of isolated hNBD1 domains,^{14–17} one school of thought suggests the primary effect of F508del arises from its role in inter-domain assembly of full length CFTR.

Nevertheless, there are also considerable *in vivo* and *in vitro* data consistent with a role for alterations in the folding efficiency and/or stability of the (F508del)hNBD1 domain itself. It has long been known that biosynthesis of (F508del)CFTR is temperature-sensitive; reducing the temperature of cell growth leads to the cell surface expression of functional (F508del)CFTR,¹⁸ while transfer back to a non-permissive temperature leads to accelerated degradation of (F508del)CFTR at the cell surface.¹⁹ The mechanism behind temperature rescue of (F508del)CFTR folding

and trafficking is due, in large part, to the improvement in physical properties intrinsic to the protein, whether it is folding efficiency, kinetics, or thermodynamic stability.²⁰ Consistent with this view, small molecule chaperones, both chemical (non-specific, e.g., glycerol) and pharmacological (showing specificity for CFTR) have been shown to mimic the effects of reduced temperature by promoting trafficking to and retention at the cell surface.^{21,22} Glycerol and other osmolytes are well known to improve protein thermodynamic stability^{23–26} and there is a solid thermodynamic and structural basis for this effect (27 and references cited within). Second site mutations in (F508del)CFTR have been identified as suppressor mutations for their ability to correct the trafficking defect.^{28–32} It is interesting that these same mutations improve the solubility of bacterially expressed hNBD1 constructs. Furthermore, the chemical chaperone glycerol improves the yield of refolded hNBD1, apparently mimicking the effects it has *in vivo* on CFTR processing and maturation.^{31,33}

Collectively, *in vivo* and *in vitro* analyses of CFTR and hNBD1 suggest that F508del perturbs either the folding efficiency (brought about by changes in the folding pathway or kinetics) and/or the native-state stability (i.e., Gibb's stabilization free energy, ΔG°) of hNBD1, independent of any subsequent inter-domain interactions of the hNBD1 domain within CFTR. However, the exact nature of this perturbation has remained unclear.^{13,15,16,32,34–36} The stabilization free energy has been assessed through isothermal chemical denaturation studies on numerous hNBD1 constructs of various sequences, mutations, and in the presence and absence of F508, and for the most part, these modifications do not alter the stabilization free energy, which is most commonly derived via linear extrapolation of the equilibrium constant for unfolding from moderate to zero denaturant concentration.^{13,15,31,37} Structures of hNBD1 domains reinforce the observations made from these stability studies. If alterations in hNBD1 stability do not account for the folding defect of (F508del)NBD1, then misfolding may arise from perturbations in the kinetics or pathway of folding/unfolding, (e.g., different intermediates may exist for (F508del)NBD1).

The apparent lack of effect of F508del on hNBD1 thermodynamic stability is the most compelling evidence against a role for the intrinsic stability of (F508del)hNBD1 on (F508del)CFTR folding and assembly, but this conclusion defies the clear effect temperature has on the folding of (F508del)hNBD1, *in vitro*^{35,36} and (F508del)CFTR, *in vivo*. However, virtually all measures of thermodynamic stability reported to date have been based on isothermal chemical denaturation methods. Furthermore, a quantitative evaluation of temperature's influence on a protein's thermodynamic stability cannot be extrapolated

Table I. Thermal Stability of Paired Human NBD1 Constructs (With and Without F508) Determined by DSC^a

Pair ID No.	hNBD1 Name ^b	Termini / Mutations ^c	T_m^d	$\Delta T_m = T_m^{\Delta 508} - T_m^{wt}$ (°C)	PDB ID
1	hNBD1-Δ(RI,RE) <i>2935c469</i> ¹⁷	387-646[Δ405-436]	57.7 ± 0.2		2PZE
1	(F508del)hNBD1Δ (RI,RE) <i>2935c472</i> ¹⁷	387-646[Δ405-436, F508del]	51.5 ± 0.3	−6.2 ± 0.3	2PZF
2		387-646[Δ405-436, V510D]	60.2 ± 0.4		
2		387-646[Δ405-436, V510D, F508del]	53.0 ± 0.1	−7.2 ± 0.4	
3		387-646[Δ405-436, F494N, Q637R]	59.2		
3		387-646[Δ405-436, F494N, Q637R, F508del]	52.8	−6.4	
4		387-646[Δ405-436, G550E, R553Q, R555K]	61.7		
4		387-646[Δ405-436, G550E, R553Q, R555K, F508del]	55.7	−6.0	
5		387-678[Δ405-436]	58.1		
5		387-678[Δ405-436, F508del]	51.7	−6.2	
6	<i>hNBD1-3</i> ¹⁵ <i>2935c382</i> ¹⁷	389-678[F429S, F494N, Q637R]	49.8 ± 0.3		
6	<i>hNBD1-3F508del</i> ¹⁵ <i>2935c371</i> ¹⁷	389-678[F429S, F494N, Q637R, F508del]	43.6 ± 0.1	−6.3 ± 0.3	2BBS
7		389-678[F429S, F494N, L636E5, Q637R]	50.5 ± 0.2		
7		389-678[F429S, F494N, L636E, Q637R, F508del]	44.9	−6.2 ± 0.2	

^a DSC conducted at 1 mg/mL protein.

^b Nomenclature for previously published constructs is cited and given in italics. Only pair 1 constructs are given an abbreviated name in the present study for convenient reference in the body of the text.

^c Expression vectors for construct pairs 1–4, 6 and 7 were obtained from SGX Pharmaceuticals, Inc. Expression vectors for construct pair 5 was obtained from Dr. Julie Forman-Kay, Hospital for Sick Children University of Toronto. Mutations are changes from the wild type human sequence and represent either deletions or point mutations of surface residues; aa 405–436 and 647–678 represent the deletions associated with the RI and RE segments, respectively.

^d T_m is defined as the temperature at the maximum in the DSC heat capacity profile, determined after subtraction of buffer baseline and progressive baseline (see Figure 2 for example of the latter data). Average T_m values, where given, represent the average of 2–3 determinations.

directly from isothermal denaturation experiments, even though the relationship between temperature (an intensive property of a system) and thermodynamic stability is clearly defined theoretically. Therefore, we set out to characterize the temperature-dependent unfolding of hNBD1 domains to evaluate explicitly the influence of F508del on their thermal stability and, by extrapolation, the temperature-dependent folding defect of (F508del)CFTR.

We present here a systematic differential scanning calorimetry (DSC) study of the thermal denaturation of a series of hNBD1 constructs, including experiments conducted at varying scan rate, hNBD1 protein concentration, and ATP concentration. Non-linear least squares fitting of these extensive experimental datasets enables us to present a thermodynamic model for the thermal unfolding of (F508del) hNBD1. The accompanying paper by Wang *et al.*³⁸ presents evidence for the first time that isothermal chemical unfolding occurs via a similar pathway. Together, these analyses have allowed us to propose an unfolding mechanism which reconciles previous disparate conclusions drawn from isothermal and ther-

mal unfolding of hNBD1. While some details of thermal and isothermal may differ, the general conclusions from both studies suggest that the hNBD1 unfolding occurs from the nucleotide-free state and demonstrate that the F508del mutation substantially destabilizes the native state and accelerates the formation of an aggregation-prone, partially unfolded intermediate. It seems likely that temperature rescue of the defective *in vivo* processing of F508del-CFTR is due, in part, to the inhibition of this unfolding pathway of the hNBD1 domain.

Results and Discussion

Deletion of F508 uniformly reduces the thermal stability of hNBD1

The thermal stability of several sequence-matched hNBD1 pairs, differing only by the presence or absence of F508, was determined by DSC (see Table I). Heat capacity profiles shown in Figure 1 for three hNBD1 pairs are typical of those obtained for all hNBD1 pairs. Table I illustrates that all (F508del)hNBD1 constructs exhibit a fairly consistent lower unfolding temperature

Figure 1. Upper Panel: DSC heat capacity profiles for hNBD1 constructs (solid lines) and the corresponding sequence-matched F508del mutant (dotted lines) in buffer containing 5 mM ATP and 10 mM MgCl₂ at 2 K/min scan rate. Buffer scans are subtracted from protein scans and data are normalized to molar concentration. Protein concentrations in the calorimetric cell were 1 mg/mL. Lower Panel: The T_m values are shown for these sequence-matched pairs together with results for the other four pairs presented in Table I. Standard errors in the T_m measurements are given in Table I.

(T_m), averaging -6.4 ± 0.4 , compared to their sequence-matched hNBD1 partner. The hNBD1 constructs studied here were identified in a screen for mutations producing sufficient expression yields and solubility for biophysical and structural studies.^{15,17} Thus, an implicit goal was to avoid alteration of the core structure in the process of improving physical behavior.

All solubilizing mutation sites are at least partially solvent exposed in natively folded hNBD1. Some of these mutations are located in disordered polypeptide segments.^{16,39} Biophysical studies on hNBD1 constructs containing suppressor mutations,¹⁵ known to partially correct the trafficking defect of cellular (F508del)CFTR that results from

misfolding,^{28–30} suggest there is a systematic linkage between improved hNBD1 solubility and improved folding and/or processing of the full length CFTR.

The regulatory insertion (RI) and regulatory extension (RE) comprise 37 and 39 residues, respectively,¹⁶ and either one (hNBD1 pair 5) or both (hNBD1 pairs 1–4) are missing in some constructs studied here. These segments are so named because they are thought to undergo regulatory phosphorylation in cellular CFTR.¹⁴ Notably, the RI and RE segments are not found in any other ABC transporters and are not required for hNBD1 folding.¹⁵ Before the reported structure of mouse NBD1,¹⁴ the RE segment was thought to be part of the R domain.⁴⁰ We previously showed that hNBD1 constructs with the RI and RE removed behave dramatically better during purification and biophysical characterization, including by DSC.¹⁷ In fact, the RI and RE segments are conformationally dynamic in solution, as determined from NMR³⁹ and hydrogen/deuterium exchange mass spectrometry.^{15,16} Therefore, these segments do not provide critical stabilizing contacts for the native hNBD1 structure. In the present work, the core structure of hNBD1 is defined as the part that does not include RI and RE. Numerous available x-ray crystal structures (see also¹³), have shown that neither the surface mutations nor the presence or absence of the RI and RE segments, noticeably alter the core structure of the protein.^{16,17}

The T_m values shown in Table I cannot be used to infer a relative thermodynamic stability for a given construct for many reasons to be discussed subsequently. Nevertheless, the T_m has been used to predict solution stability at temperatures considerably lower than T_m . It is interesting that deletion of RI has the most dramatic effect on T_m when compared to the other solubilizing mutations studied here, illustrated in the bar graph of Figure 1 (e.g., compare hNBD1 pair 5 with pairs 6 and 7) and suppressor mutations further increase the thermal stability, although to a more limited extent (compare hNBD1 pair 1 or 5 with pairs 2, 3 or 4). The Teem suppressor triplet (pair 4)²⁹ increases T_m by 4°, the V510D mutation (pair 2)³⁰ by 2.5°, and F494N/Q637R (pair 3) by 1.5°. These observations suggest the basis for their improved solubility may involve alteration of the unfolding kinetics and/or thermodynamics, rather than solely influencing the propensity of the folded protein to self-associate, as suggested previously.¹⁵

Consistent with the possibility of a mechanistic coupling between domain solubility and unfolding kinetics and/or thermodynamics, the F508 deletion, which we will later show reduces the thermodynamic stability of hNBD1-Δ(RI,RE), also reduces the solubility of all hNBD1 constructs.^{15,17} The largely invariant reduction in T_m of about 6–7°C suggests its deletion causes the same change in the folding/

unfolding pathway of all the hNBD1 constructs. Likewise, the energetic consequences of the surface mutations, and the presence or absence of the regulatory segments, appear to be largely independent of the energetic consequences of the presence or absence of F508. This observation is consistent with an earlier analysis of a comprehensive set of NBD1 crystal structures suggesting no direct or allosteric coupling between the various mutations sites.¹⁶ Furthermore, the view that the NBD1 mutations reported here similarly affect the thermodynamic and/or kinetics of NBD1 unfolding in the presence and absence of F508del is directly addressed in the accompanying paper by Wang *et al.*³⁸ They present the results of chemical denaturation studies for several hNBD1 pairs in Table I and demonstrate that for a given set of solubilizing mutations, similar effects on chemical denaturation are observed with and without the F508del mutation.

Thermal unfolding of hNBD1 is irreversible and kinetically-controlled

We chose to focus further DSC analysis on the hNBD1-Δ(RI,RE) construct pair since crystal structures exist for this pair and they have otherwise been well-characterized.¹⁷ The differences in thermodynamic stability of these domains can be determined through a comparison of their Gibbs stabilization free energy, ΔG° , and DSC directly provides the parameters needed to calculate stabilization free energy at any temperature. However, a thermodynamic treatment of DSC data is only valid if thermal unfolding is an equilibrium process. Equilibrium is typically inferred if the unfolding transition is reversible (i.e., seen on a second heating scan). Furthermore, the data obtained must be independent of the DSC scan rate.

Unfortunately, the DSC transition is irreversible. Even under the best conditions, less than 20% reversibility is seen (data not shown). The simplest model to account for an irreversible unfolding process is the three-state model, $N \rightleftharpoons U \rightarrow D$. This model was first proposed by Lumry and Eyring to describe the irreversible unfolding of proteins.⁴¹ According to this simple model, an equilibrium exists between the folded and unfolded states, N and U, which is followed by the irreversible formation of D, a species which is unable to refold within the time frame of the DSC experiment.

The unfolding transition temperature for both hNBD1-Δ(RI,RE) constructs is also seen to vary with scan rate (shown in Supporting Information Fig. 1). This indicates that the rate of D formation is fast compared to all scan rates tested. Therefore, under the conditions of the DSC experiment, thermal unfolding is a kinetically-controlled process and, the scan-rate dependency of this process provides information on the rate of D formation.

Table II. DSC Transition T_m and ΔH_{cal} for Unfolding of hNBD1-Δ(RI,RE) and (F508del)hNBD1-Δ(RI,RE)^a

Protein (mg/mL)	T_m (°C)	
	hNBD1	(F508del)hNBD1
0.5	57.8	51.1
1	57.9	50.7
2	57.8	51.2
4–5	57.7	51.2
9–10	57.9	51.3
Average $T_m \pm SD$	57.8 ± 0.1	51.1 ± 0.2
Average ΔH_{cal} (kcal/mol) ^b	89.7 ± 4.3	76.3 ± 5.7

^a Performed at scan-rate 2°C/min at 5 mM ATP and 10 mM MgCl₂.

^b ΔH_{cal} is the integrated area of the DSC endothermic transition normalized to total moles of protein

In fact, there is a strong theoretical foundation for extracting both kinetic and thermodynamic parameters from DSC scan-rate dependent data^{42–45} and these concepts have been successfully applied to numerous systems.^{46–50} It has been shown that thermodynamic parameters associated with the $N \rightleftharpoons U$ equilibrium step, as well as kinetic parameters for the irreversible step $U \rightarrow D$ can be obtained (i.e., the rate constant, k_{irr} , and activation energy E_a). However, when k_{irr} is sufficiently faster than the rate of conversion of U back to N, then N and U are no longer in equilibrium. In this case, the unfolding reaction collapses to a two-state irreversible process between the native and denatured states, denoted by $N \rightarrow D$. Consequently, without further analysis of the DSC data, the unfolding mechanism for the thermal unfolding of hNBD1 could be represented by either $N \rightleftharpoons U \rightarrow D$ or $N \rightarrow D$.

The DSC unfolding transition of hNBD1 is independent of protein concentration

Aggregation is a common factor that results in irreversible unfolding. Therefore, it was necessary to determine whether or not changes in the protein concentration altered either the T_m or integrated area (ΔH_{cal}) of the DSC unfolding transition. If aggregation occurs during the formation of D (in contrast to *after* the formation of D), then the rate constant for its formation, k_{irr} , will be at least second order (e.g., in the case where a dimer is formed), if not higher. In this case, increasing protein concentrations will lead to faster rates of D formation and a lowering of the T_m .⁴³ Even under ideal buffer conditions, isolated hNBD1 domains do not maintain solubility over prolonged periods and it is not surprising that thermal unfolding leads to aggregation that is very obvious when monitored by light scattering (see accompanying paper). Thus it was surprising to see no dependence on protein concentration of either the transition T_m or ΔH over a twenty-fold range of concentration (Table II). Even at temperatures above the DSC transition there was no heat

Figure 2. CD spectra of hNBD1-Δ(RI,RE) and (F508del)hNBD1-Δ(RI,RE) in the far-UV region (left panel) and in the near-UV region (right panel). Solid circles are the experimental data for hNBD1-Δ(RI,RE) and open circles are the experimental data for (F508del)hNBD1-Δ(RI,RE). The ATP concentration for far-UV measurements was 0.8 μ M for (hNBD1-Δ(RI,RE)) and 4 μ M ATP for (F508del)hNBD1-Δ(RI,RE); for near-UV measurements, it was 0.125 mM ATP for both proteins. Protein concentration was 0.69 mg/mL in 0.02 cm cuvettes for far-UV CD and 1 mg/mL in 1 cm cuvettes for near-UV CD measurements.

change or detectable deviation in the instrument baseline, as is sometimes observed when post-transition aggregation leads to precipitation (see Fig. 1). A post-transition, aggregation-related, heat effect is most likely absent in the DSC heat capacity profile because of the improved cell geometry of the DSC instrument used (see Methods for further details).

Nevertheless, the significant observation from the protein concentration study is that for both hNBD1-Δ(RI,RE) constructs, aggregation occurs *after* formation of the rate-limiting product of thermal unfolding, which must therefore be monomeric D.^{44,47} A similar mechanism has been proposed for the aggregation properties of partially unfolded recombinant human interferon- γ .^{44,51} To better represent the concept that D is an aggregation-prone species produced from thermal unfolding, the acronym A_T will be used in place of D from this point forward. Therefore, at this point, the likely thermal unfolding mechanisms for hNBD1 are represented by either $N \rightleftharpoons U \rightarrow A_T$ or $N \rightarrow A_T$.

Circular dichroism (CD) spectroscopy provides evidence that A_T , the product of thermal unfolding, is partially folded

Changes in the secondary and tertiary structures of the hNBD1-Δ(RI,RE) pair during thermal unfolding were investigated by monitoring far- and near-UV CD, respectively, to shed light on possible differences that may account for differences in their thermal stabilities. CD spectra of the hNBD1-Δ(RI,RE) pair are shown in Figure 2. It has been shown that the deletion of F508 produces only local conformational changes near the site of the mutation in the crystal structures of various (F508del)hNBD1 constructs; the structure is otherwise the same for all available structures of hNBD1.^{13–17} Therefore it is not surpris-

ing that the CD spectra are very similar, if not identical for hNBD1-Δ(RI,RE) and (F508del)hNBD1-Δ(RI,RE). Some difference in the near-UV CD spectra is not unexpected, since the protein residues that primarily contribute to the near-UV CD contain aromatic side chains, like phenylalanine, which absorbs between about 240–275 nm. Absorption of bound MgATP could also contribute in this region and thereby account for some minor variability in the near-UV CD region, which was noted depending on the ATP concentration. That is, differences in the MgATP binding constant for the two constructs could contribute to the observed differences in the spectrum near about 260 nm. It is also possible that bound ATP influences the protein spectrum, itself, for example, arising from its interaction with the tryptophan 401 side chain, which stacks against the adenine ring of ATP.¹⁵

Because thermal unfolding is influenced by the temperature scan rate, DSC and CD unfolding data were collected at the same scan rate, 2°C/min. For ease of comparison, all data are normalized to the fractional change observed (Fig. 3). Therefore, the DSC transitions have been integrated, which results in the sigmoidal progress curves seen in Figure 3. The noteworthy observations are that qualitatively similar behavior is observed in the presence or absence of the F508del mutation and the temperature-dependent loss in near-UV CD ellipticity is superimposable on the integrated DSC unfolding transition. In contrast, the major change in the far-UV CD occurs at temperatures significantly above the DSC transition, where there is no detectable change in heat capacity.

For the purpose of comparison to data collected by isothermal chemical denaturation (see accompanying paper by Wang *et al.*³⁸), the change in

Figure 3. Thermal unfolding of hNBD1- Δ (RI,RE), left panels, and (F508del)hNBD1- Δ (RI,RE), right panels, monitored by CD and DSC at scan rate 2°C/min at 0.125 mM ATP and 0.5 mM MgCl₂. CD is monitored at 297 nm (near-UV wavelength region, upper panels) and at 230 nm (far-UV wavelength region, lower panels). Protein concentration in the 1 cm cuvette was 0.125 mg/mL for far-UV and 0.5 mg/mL for near-UV measurements. The unfolding data were normalized to the fractional change observed. No baseline subtraction was performed on the spectral data shown by closed and open circles. DSC unfolding curves (represented by solid lines in the upper panels) were integrated and normalized to the unfolding calorimetric enthalpy value, $\Delta H_T/\Delta H_{cal}$; CD unfolding curves were normalized to maximal change in the CD signal, $CD_T/(CD_{initial}-CD_{final})$, where T = temperature of observed signal. The signal change at 230 nm for both proteins was approximately the same: starting at 20°C, $\theta = -6 \times 10^3 \text{ deg cm}^{-1} \times \text{dmole}^{-1}$ and ending at 80°C, $\theta = -2 \times 10^3 \text{ deg cm}^{-1} \times \text{dmole}^{-1}$. The signal change at 297 nm for hNBD1- Δ (RI,RE) was determined from the initial signal at 20°C, $\theta = 69 \times 10^3 \text{ deg cm}^{-1} \times \text{dmole}^{-1}$ and ending at 60°C, $\theta = -58 \times 10^3 \text{ deg cm}^{-1} \times \text{dmole}^{-1}$; for (F508del)hNBD1- Δ (RI,RE), initially, $\theta = 55 \times 10^3 \text{ deg cm}^{-1} \times \text{dmole}^{-1}$ and ending with $\theta = -31 \times 10^3 \text{ deg cm}^{-1} \times \text{dmole}^{-1}$. The insets in the upper panels are temperature unfolding of hNBD1- Δ (RI,RE), left panel, and (F508del)hNBD1- Δ (RI,RE), right panel, monitored by intrinsic Trp fluorescence with excitation wavelength at 290 nm and emission wavelength at 340 nm and DSC at scan rate 1°C/min at 0.1 mM ATP and 0.2 mM MgCl₂. Trp fluorescence unfolding curves were normalized to maximal change in the Trp fluorescence signal, $FL^{Trp}_T/(FL^{Trp}_{initial}-FL^{Trp}_{final})$, where T = temperature of observed signal. Preunfolding and postunfolding baselines of unfolding profile were subtracted using equations obtained by linear regression to obtain the data shown.

intrinsic fluorescence was also monitored (Fig. 3, insets). Similar to the near-UV CD signal, a cooperative increase in emission at 340 nm appears coincident with the DSC transition, suggesting a change in the environment surrounding the tryptophan residues.

After heating to 80°C, the protein samples were cooled to room temperature and spectra were taken. While there is some remaining ellipticity in the sample, which is typical for thermally unfolded proteins, the spectra in both the far-UV and near-UV CD were devoid of the spectral characteristics identifiable as native-like structure (data not shown). A loss

of essentially all the near-UV CD signal and a fractional loss of about 20% of the secondary structure coincide with the only thermal unfolding transition detected by DSC, indicating that A_T exhibits appreciable residual structure.

Thermal unfolding of hNBD1 is strongly influenced by equilibrium binding of MgATP

Because hNBD1 constructs have improved solubility in the presence of MgATP, all studies described so far have been in the presence of at least nominally saturating amounts of MgATP. The quantitative

Figure 4. DSC of hNBD1-Δ(RI,RE) and (F508del)hNBD1-Δ(RI,RE) at different concentrations of ATP at scan-rate 2 K/min. The left panel shows the temperature dependence of the molar heat capacity of (hNBD1-Δ(RI,RE) (solid lines) and (F508del)hNBD1-Δ(RI,RE) (dotted lines) at 0 mM, 0.1 mM and 5 mM ATP. The right panel represents results of DSC data analysis for hNBD1-Δ(RI,RE) (solid circles) and (F508del)hNBD1-Δ(RI,RE) (open circles) as ATP concentration dependence of T_m (top panel) and unfolding enthalpy (bottom panel) obtained from 0 mM to 20 mM ATP. Protein concentration was 1 mg/mL. The minimal detectable ATP concentration in the protein solution is 0.5 μ M; see Supporting Information Methods for details.

effects of MgATP binding on thermal stability were, therefore, determined, and are shown in Figure 4. T_m and ΔH_{cal} steeply increase at low concentrations and then rise more slowly at concentrations above complete hNBD1 saturation.

A manifestation of Le Chatelier's Principle is the widely observed increase in protein unfolding T_m in the presence of ligand binding. Unfolding occurs at higher T_m because ligand binding to the folded protein shifts the $N \rightleftharpoons U$ equilibrium in favor of the native state, N. Therefore, the observed influence of MgATP binding on hNBD1 thermal unfolding suggests that of the two unfolding mechanisms proposed above, the more likely is $N \rightleftharpoons U \rightarrow A_T$. In the presence of MgATP the equilibrium between bound and free hNBD1 must also be included, that is, $MgATP \cdot N \rightleftharpoons N + MgATP$.

Because we know from our CD studies that A_T is only partially unfolded, we propose that the $N \rightleftharpoons U$ equilibrium in the overall unfolding process is better represented by $N \rightleftharpoons I_T$, where I_T denotes a species with a conformation intermediate between N and A_T . Thus, the minimum unfolding pathway that is likely to represent the thermal unfolding of hNBD1-Δ(RI,RE) is $MgATP \cdot N \rightleftharpoons N \rightleftharpoons I_T \rightarrow A_T$. This pathway is referred to as Model ii in subsequent discussions (see Table III).

DSC curve fitting is consistent with the $N \rightleftharpoons I_T \rightarrow A_T$ model, which includes MgATP binding equilibria to both N and I_T

Two sets of DSC data were collected for each protein; the first comprising experiments at 2 mM MgATP with scan-rate varying between 0.5 – 2 degree/min (Supporting Information Fig. 1) and the second comprising experiments at a constant scan rate of 2

degree/min with MgATP concentration varying between 0.088 and 10 mM for hNBD1-Δ(RI,RE) and 0.088–5 mM for hNBD1-Δ(RI,RE)-F508del (Fig. 5). For each mechanistic model used for curve-fitting (shown in Table III), the scan-rate dependent datasets were globally fit first to obtain the best fits for the kinetic parameters for formation of A_T (see Table IV for fitted kinetic parameters). Subsequently, the MgATP concentration-dependent datasets were globally fit to determine the parameters for the equilibrium step(s), while constraining the kinetic parameters to a narrow range of values around the results from the first fits. The equations used to fit each model and further details on the fitting procedure can be found in the Supporting Information Materials and Methods.

We evaluated the mechanistic models based on three criteria, summarized in Supporting Information Table I. First, we compared the χ^2 values for

Table III. Hypothetical Unfolding Mechanisms used to Fit DSC Data^a

Model	Unfolding pathway
i	$MgATP \cdot N \rightleftharpoons N \xrightarrow{MgATP} A_T \rightarrow (A_T)_n$
ii	$MgATP \cdot N \rightleftharpoons N \rightleftharpoons I_T \rightarrow A_T \rightarrow (A_T)_n$
iii(a)	$MgATP \cdot N \rightleftharpoons N \rightleftharpoons I_T \rightarrow A_T \rightarrow (A_T)_n$
	$MgATP \cdot I_T$
iii(b)	$MgATP \cdot L \rightleftharpoons I_T \xrightarrow{MgATP} A_T \rightarrow (A_T)_n$
	$MgATP \cdot N \rightleftharpoons N \xrightarrow{MgATP} A_T \rightarrow (A_T)_n$

^a Step in brackets, $[--->(A_T)_n]$, is not fit by the mathematical models; for details of the curve fitting, see Materials and Methods

Figure 5. Comparison of global fits of DSC data to Models i, ii, and iii(a). Left panels: Fits for hNBD1- Δ (RI,RE). Right panels: Fit for (F508del)hNBD1- Δ (RI,RE). DSC was performed at scan rate 2°C/min in buffer containing 0.088, 0.108, 0.196, 0.3, 0.46, 0.915, 0.97, 1.82, 4.7 mM ATP with 10 mM MgCl₂, and 10 mM ATP with 20 mM MgCl₂ for hNBD1- Δ (RI,RE) and 0.088, 0.11, 0.21, 0.27, 0.48, 0.85, 1.05, 1.7 and 4.9 mM ATP with 10 mM MgCl₂ for (F508del)hNBD1- Δ (RI,RE). Solid lines are experimental data. Dashed lines are best fit curves.

the fitted curves; the smaller the value, the better the fit. The χ^2 for the fit to a given model is seen to decrease as the complexity and associated number of fitting parameters increase, that is, from Model i to Model iii(b), χ^2 becomes progressively smaller. However, the better fit may only be the result of the greater number of fitting parameters, and would not necessarily mean the model with the lowest χ^2 is the correct model. Therefore two other methods were used to discriminate among the models, the statistical F-test and the Akaike's Information Criterion (AIC), based on information theory. These evaluation methods take into account the number of fitting pa-

rameters in computing the relative probability that a given model is the best fit. The detailed methods for quantitative comparison of F-ratios and the AIC can be found in the legend to Supporting Information Table I and Materials and Methods. From this analysis, Model ii was clearly better than Model i. In Model ii, the I_T state does not bind MgATP; however there is no structural or mechanistic reason to rule out ATP binding by state I_T , so a model incorporating ATP binding to I_T , Model iii(a), was tested and the fit was improved further and also deemed to be more likely correct based on both the F-ratio and the AIC. The fitted curves for Models i, ii and iii(a)

Table IV. Best Fit Parameters for Unfolding Mechanism Model iii(a) from Global Fit of DSC Data^a

Process	Parameters	hNBD1-Δ(RI,RE)	(F508del)hNBD1-Δ(RI,RE)
	χ^2	2.9E + 04	5.9E + 04
$N \rightleftharpoons I_T$	Ku at 45°C	1.0 ± 0.04	2.8 ± 0.6
$\text{MgATP} \bullet N \rightleftharpoons N + \text{MgATP}$	$H_{v,r}$ at respective T_m (kcal/mol)	70.6 ± 2.5	57.2 ± 3.9
	K_{d1} at 25 °C (μM)	0.51 ± 0.16	1.0 ± 0.5
	H_{d1} at 25 °C (kcal/mol)	20.6 ± 2.8	25.6 ± 5.0
$\text{MgATP} \bullet I_T \rightleftharpoons I_T + \text{MgATP}$	K_{d2} at 52.8 °C (μM)	$(6.1 \pm 0.2) \times 10^3$	$(2.5 \pm 0.5) \times 10^3$
	H_{d2} at 52.8 °C (kcal/mol)	-6.9 ± 2.0	-10 ± 4.2
	k_{irr} at 58 °C (min^{-1})	3.1 ± 0.5	11.5 ± 6.9
$I_T \rightarrow A_T$	E_a (kcal/mol)	33.5 ± 1.0	23.9 ± 2.3
	$H_{d,irr}$ at 58 °C (kcal/mol)	-15.8 ± 1.1	-18.4 ± 2.5

^a Estimated standard errors in each parameter value are based on 95% confidence level.

are shown with the DSC data in Figure 5. A variant of Model iii(a), denoted iii(b), switches $N \rightleftharpoons I_T$ from an on-pathway (with respect to A_T formation) to an off-pathway step. This variant was suggested from the early work of Thomas and coworkers³¹ who suggested that the formation of an aggregation-prone intermediate may not be on the unfolding pathway. The χ^2 value for Model iii(b) is similar to that for Model iii(a). However, F-test and AIC analysis suggests Model iii(b) is, by far, less likely to be correct than Model iii(a). Incorporation of additional states (and the requisite additional parameters) into the unfolding model did not result in improved fits based on any of our evaluation criteria and were mechanistically unnecessary to account for the unfolding data.

We conclude that of the mechanistic models tested, Model iii(a) is the most likely to represent the thermal unfolding mechanism for hNBD1. Table IV gives the values obtained for the parameters fit to this model and Supporting Information Figure 2 shows plots of the fraction of each species present as a function of temperature at two different ATP concentrations.

Of critical importance for any proposed model, the values obtained for all parameters must be physically reasonable and this is confirmed for Model iii(a) as shown in Table IV. The fitted value for hNBD1-Δ(RI,RE) binding to MgATP, $K_{d1} = 0.5 \mu\text{M}$ at 25°C and $0.04 \mu\text{M}$ at 4°C, are within the range of the value measured by surface plasmon resonance at 4°C, $0.8 \mu\text{M}$.¹⁷ The corresponding fitted value for (F508del)hNBD1-Δ(RI,RE) is similar. There is no published value for this construct but for a different sequence pair, the binding constants for NBD1 and for (F508del)NBD1 are similar.³¹ The remaining parameters will be discussed in more detail below.

Conclusions and Interpretations

Taken on face value, these results represent a new perspective on hNBD1 stability and provide significant insights into the unfolding mechanism. The

new information provided by these studies are summarized as follows: (1) the native state of (F508del)hNBD1 is thermodynamically less stable than hNBD1; (2) an intermediate, I_T , exists in the unfolding pathway and retains weak affinity for MgATP; (3) a kinetically-trapped, partially unfolded state, A_T , is the aggregation-prone conformational state in hNBD1 thermal unfolding; (4) lower temperature will reduce the extent of hNBD1 unfolding and the aggregation resulting from this pathway; (5) since temperature affects CFTR folding, it is reasonable to suggest that in the context of the CFTR protein, reduced-temperature rescue of folded (F508del)hNBD1 results from less A_T formation and consequently less non-native and counter-productive interactions with other CFTR domains; and (6) all the unfolding models tested here support the proposal that lower temperature will reduce the aggregation resulting from hNBD1 unfolding in a quantitatively predictable manner. The latter inference is supported by the comparison of the kinetic parameters for the formation of A_T derived by each model shown in Table III. Regardless of the model used, the derived rate, k_{irr} , for formation of A_T is significantly faster for (F508del)hNBD1-Δ(RI,RE) (see Supporting Information Table I). As illustrated in Figure 6, as temperature is lowered from T_m to below 37°C, the rate of A_T formation slows dramatically.

Before the studies presented here, and in the accompanying paper,³⁸ all published chemical denaturation studies have been performed at a fixed temperature, near ambient. In those studies, the observed unfolding reaction has been modeled as a simple, two-state transition between a native and unfolded state and a stabilization free energy, ΔG° , was determined by linear extrapolation of ΔG from moderate denaturant concentration to 0 M denaturant. The difference in ΔG° ($\Delta\Delta G^\circ$) between hNBD1 and (F508del)hNBD1 determined from those previous studies was negligible ($\ll 1$ kcal/mol), giving rise to the interpretation that F508del does not alter the stability of hNBD1. From those studies, the ΔG° values for hNBD1 have ranged, depending on the

Figure 6. Temperature-dependence of the rate constant, k_{irr} , for the $I_T \rightarrow A_T$ unfolding step for hNBD1- Δ (RI,RE) and (F508del)hNBD1- Δ (RI,RE). Fitted parameters in Table IV were used to construct the plot.

construct, from about 3.7 kcal/mol^{13,37} to 11 kcal/mol.¹⁵ On the other hand, we show that thermal unfolding is under kinetic control and is clearly not two-state. As we will show in the accompanying paper,³⁸ a re-examination of hNBD1 chemical denaturation suggests it is also not two-state.

For the present studies, the only thermodynamically valid unfolding ΔG° one may calculate is for the $N \rightleftharpoons I_T$ step; $\Delta G^\circ = 3.2$ kcal/mol and 2.1 kcal/mol at 25°C, for hNBD1- Δ (RI,RE) and (F508del)hNBD1- Δ (RI,RE), respectively. Because I_T is not completely unfolded nor is it the final unfolded species produced by thermal denaturation, it is not surprising that these values are rather small. However, the difference between them, which is $\Delta\Delta G^\circ = 1.1$ kcal/mol, is larger than the negligible difference found in previous chemical denaturation studies (by as much as fourfold). Furthermore, the fractional difference is even bigger, that is, (F508del)hNBD1- Δ (RI,RE) is about one third less stable than hNBD1- Δ (RI,RE).

Qu and Thomas³⁷ were the first to report a modest 3°C difference in the T_m between protein constructs containing an incomplete hNBD1 domain in the absence of presence of the F508del mutation, but they discounted the significance of this difference, given the inconsequential $\Delta\Delta G^\circ$ they obtained by chemical denaturation. There is a two-part explanation for the unexpectedly large difference in thermal stabilities that we see in the present studies between complete hNBD1 domain constructs in the absence of presence of the F508del. As shown in Figure 7, for (F508del)hNBD1- Δ (RI,RE), both ΔH and ΔS are smaller for the $N \rightleftharpoons I_T$ unfolding step, which translate a modest $\Delta\Delta G^\circ$ at 25°C into a demonstrably significant thermal stability difference. That is, there is a 4 degree difference in T_m (read from the temperature at which $\Delta G = 0$ for each construct).

Figure 7. Plots of ΔG , ΔH , and $T\Delta S$ versus temperature for the $N \rightleftharpoons I_T$ transition. The fitted ΔC_p values of 1.86 kcal/mol/K, and 2.13 kcal/mol/K, for hNBD1- Δ (RI,RE) and (F508del)hNBD1- Δ (RI,RE), respectively, with other fitted parameters found in Table IV were used to construct the plots.

The faster rate of the irreversible step $I_T \rightarrow A_T$ also independently lowers T_m for (F508del)hNBD1- Δ (RI,RE). Together these two unfolding steps produce the observed ΔT_m of $\sim 6 - 7^\circ\text{C}$. As a result, significantly more I_T and A_T are formed for (F508del)hNBD1- Δ (RI,RE) at every temperature. For instance, at 37°C, 3.3 times more I_T is formed, which is irreversibly converted to A_T with a rate that is 10 times faster than the rate for hNBD1- Δ (RI,RE) (see Fig. 6). As the temperature increases, the formation of I_T and the rate of A_T formation also rise, resulting in more and more A_T formation at higher temperatures.

Given conclusions (5) and (6) above, it is tempting to speculate that the A_T conformation identified in these studies is equivalent to the aggregation-prone, partially folded state previously proposed by Thomas and coworkers³¹ and now characterized in detail in the isothermal chemical denaturation studies presented in the accompanying paper by Wang *et al.*³⁸ It is also possible that the loss of secondary structure observed *above* the DSC transition temperature represents the major transition observed in chemical denaturation. In fact, the results we present in Wang *et al.*³⁸ support this hypothesis. We show that under conditions of low MgATP concentration, chemical denaturation is most likely represented by a two-step unfolding pathway, a simplified representation of which is given by $\text{MgATP} \cdot N \rightleftharpoons N \rightarrow [A_C]_n \rightarrow U$. We propose that the structural transition of the first step in isothermal chemical denaturation is similar if not identical to the structural transition observed by DSC. This step leads to only a minor loss in average secondary structure and the formation of a partially folded, aggregation-prone intermediate, A_C . The second step of chemical denaturation is likely to be similar to the thermal transition

following that detected by DSC, which does not involve a significant enthalpy change, but as with chemical denaturation, produces a species with minimal secondary structure as monitored by CD. It is tempting to speculate³⁸ that A_C and A_T share some properties that are predicted for a molten globule, for example, presence of secondary structure but no apparent tertiary structure (as probed by CD) and a low structural cooperativity resulting in the absence of a calorimetric transition.^{52–54} The full details and experimental support for the isothermal chemical denaturation pathway, and the associated structural transitions proposed for hNBD1, are presented in our accompanying paper.³⁸

In summary, the studies reported here represent the first thermodynamic description of hNBD1 thermal unfolding. One plausible explanation exists for the increased solubility of all “solubilizing” mutations, regardless of origin. While improvement in solubility could arise from the properties of the folded domain, in all cases studied here, the likely alternative explanation is that aggregation is initiated from the partially unfolded A_T state, not the folded state, and solubilizing mutations act by inhibiting the formation of A_T . Furthermore, the inclusion of high MgATP concentration or osmolytes like glycerol and other so-called “stabilizing” additives lead to improved solubility by inhibiting the formation of A_T .

These studies may also provide a highly relevant model for the thermal stability of CFTR during its biosynthesis, folding, and domain assembly. It is likely that temperature- and chaperone-rescue of the (F508del)CFTR trafficking defect results from the increased thermodynamic stability of the (F508del) hNBD1 domain and the reduced rate of formation of an aggregation-prone, partially folded (F508del) hNBD1 domain. The correlation between improved hNBD1 domain solubility and improved folding and/or processing of full length CFTR that has been observed in the presence of suppressor mutations may be likewise explained, and has been recently extended to a study of (F508del)CFTR containing the RI deletion.⁵⁵

Material and Methods

Protein purification

Protein purification was conducted as previously described,¹⁵ yielding protein in 150 mM NaCl, 20 mM HEPES pH 7.5, 10% glycerol, 10% ethylene glycol, 1 mM Tris(2-carboxymethyl)phosphine (TCEP), 2 mM ATP, 3 mM magnesium chloride. Proteins were >98% pure as judged by Coomassie Blue staining of SDS-PAGE gels, showed no evidence of aggregation and ran as monomers during gel filtration. Protein concentration in the absence of ATP was measured spectrophotometrically using an extinction coefficient for hNBD1- Δ (RI,RE) equal to 0.9 OD_{280 nm}/mg/mL, and

in the presence of ATP concentration, was measured with the Bio-Rad Protein assay in microplate format, calibrated using *B. subtilis* NAD synthetase.

Differential scanning calorimetry (DSC)

Calorimetry was carried out on the Nano-DSC II (CNC, U.S.A.) in 0.299 mL cells or on the VP-Capillary DSC System (MicroCal Inc., GE HealthCare, U.S.A.) in 0.130 mL cells at heating rates from 0.5 K/min to 2 K/min. An external pressure of 2.0 atm was maintained during all DSC runs to prevent possible degassing of the solutions on heating. Unless otherwise indicated, the buffer for DSC and CD experiments was 20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM TCEP, 10% glycerol and 10% ethylene glycol, containing ATP at up to 20 mM (as noted) and MgCl₂ up to 40 mM (as noted). The reversibility of unfolding was checked by sample reheating after cooling in the calorimetric cell. The heat capacity curves were corrected for the instrumental baseline. DSC data were analyzed with the MicroCal Origin software, from which the unfolding temperature (T_U), and the calorimetric (ΔH_{cal}) and van't Hoff (ΔH_{vH}) unfolding enthalpies were obtained. Mathematical equations describing possible models were written in a format recognizable by Origin, based on the equations described under DSC analysis, and used for non-linear least-squared curve-fitting.

The DSC cells used for these studies have a capillary design, which is known to help minimize the heat effects accompanying sample precipitation. This seems to be the most likely explanation for the lack of post-transition baseline effects (see Fig. 1). Heat capacity profiles of hNBD1- Δ (RI,RE) acquired with a DSC instrument equipped with cylindrical cells, exhibit a post-transition baseline anomaly, consistent with the precipitation event (data not shown).

Circular dichroism (CD)

CD spectra were recorded on an Olis RSM 1000 CD spectrophotometer (OLIS) or a CD Spectrophotometer (JASCO, Japan) equipped with the thermostatic platform and programmable circulating water bath. The cells had a path length of 0.02 and 1.0 cm, respectively, for far-UV spectra at 0.5–1 mg/mL and for near-UV spectra at 0.8 – 1.6 mg/mL. Spectra were recorded from 260 to 200 nm for far-UV and from 320 to 260 nm for near-UV in 0.5-nm steps. The molar ellipticity values were calculated according to the following expression: $[\Theta] = (\Theta) (100 \times MRW/lc)$, where Θ is the measured ellipticity in degrees, MRW is the mean residues molecular weight in grams per mole (111.3 and 111.2 for hNBD1- Δ (RI,RE) and (F508del)hNBD1- Δ (RI,RE), respectively), l is the path length in centimeters, and c is the concentration of the protein in grams per liter. The value $[\Theta]$ has the units of degrees•cm²/decimole-residue. For protein unfolding monitored by CD, a

cell with a path length of 1.0 cm was used. The temperature was increased at a scan rate of 1 or 2 °C/min, the sample solution was stirred at 250 rpm, and the CD intensity was recorded continuously at ~230 nm or ~290 nm.

Intrinsic fluorescence

A PolarStar Optima (BMG LABTECH) fluorescence plate reader was equipped with 290 ± 5 nm excitation and 340 ± 5 nm emission filters and an integrated heating block fabricated to hold a 384 microtiter plate. Thermal protein unfolding was conducted at a 1°C/min heating rate and monitored by following the change in intrinsic fluorescence intensity at 340 nm. Due to technical limitations, CD, DSC, and fluorescence could not all be monitored at the same scan rate. Experiments were conducted at 0.4 mg/mL protein concentrations in 20 mM HEPES pH 7.5, 10% glycerol, 10% ethylene glycol, 150 mM NaCl, 0.1 mM ATP, 0.2 mM MgCl₂, 1 mM TCEP; 15 µL oil (chill-out liquid clear, Bio-Rad Lab, Inc.) was placed over the 30 µL sample to prevent evaporation during heating. Data were collected and analyzed using in-house software written for this purpose.

DSC data analysis by nonlinear curve fitting

Data were fit using the non-linear regression algorithm of Levenberg-Marquardt with Origin 7.0 (OriginLab Corporation, Northampton, MA 01060). Origin C, a programming language included in Origin, was used to write mathematical equations describing the various unfolding models. For details, see Supporting Information Material and Methods.

Quantitative discrimination among models used to fit the DSC data

A comparison of models was made using statistical methods, that is, goodness-of-fit as quantified by the residual χ^2 , the F test, and a method based on information theory, Akaike's Information Criterion, (AIC). For a description of all of these methods see reference.⁵⁶ The F test can only be applied to nested models, that is, in cases where the complex model containing more parameters can be transformed into the simpler model by setting one or more of the parameters to either 0 or 1. The AIC can be used to compare both nested and non-nested models. Details on how these methods were applied can be found in the Supporting Information Materials and Methods.

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