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Temperature Dependent Hammond Behavior in a Protein Folding Reaction: Analysis of Transition State Movement and Ground State Effects

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Summary

Characterization of the transition state ensemble and the nature of the free energy barrier for protein folding are areas of intense activity and some controversy. A key issue which has emerged in recent years is the width of the free energy barrier and the susceptibility of the transition state to movement. Here we report denaturant and temperature dependent folding studies of a small mixed α - β protein, the N-terminal domain of L9 (NTL9). The folding of NTL9 was determined using fluorescence detected stopped-flow fluorescence measurements conducted at seven different temperatures between 11 and 40 °C. Plots of the log of the observed first order rate constant vs. denaturant concentration, “chevron plots”, displayed the characteristic V-shape expected for two state folding. There was no hint of deviation from linearity even at the lowest denaturant concentrations. The relative position of the transition state, as judged by the Tanford β parameter, β_T , shifts towards the native state as the temperature is increased. Analysis of the temperature dependence of the kinetic and equilibrium m-values indicates that the effect is due to significant movement of the transition state and also includes a contribution from temperature dependent ground state effects. Analysis of the Leffler plots, plots of $\Delta G^\ddagger$ vs. ΔG° , and their cross-interaction parameters confirm the transition state movement. Since the protein is destabilized at high temperature, the shift represents a temperature dependent Hammond effect. This provides independent confirmation of a recent theoretical prediction. The magnitude of the temperature-denaturant cross-interaction parameter is larger for NTL9 than has been reported for the few other cases studied. The implications for temperature dependent studies of protein folding are discussed.

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Supplementary Materials

A plot of β_T corrected for ground state effects vs. $\Delta G^\circ T$.

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Keywords

protein folding; rate equilibrium free energy relationship; Leffler plot; Hammond effect; ribosomal protein L9

Introduction

Characterization of the transition state ensemble is a key step in developing a detailed, quantitative picture of protein folding, particularly for proteins which fold in a two-state fashion.^{1; 2; 3; 4} One key issue which has emerged in recent years is the susceptibility of the transition state to movement and the width of the free energy barrier. Experimental investigations of the transition state often make use of rate equilibrium free-energy relationships (REFERs). In many cases a linear relationship is observed between changes in the activation free energy and changes in the equilibrium free energy of the system. This relationship is defined by the equation:

$$\alpha_x = \left(\frac{\partial \Delta G^\ddagger}{\partial x} \right) / \left(\frac{\partial \Delta G^0}{\partial x} \right) \quad (1)$$

α_x is defined as the interaction parameter where the subscript x represents the type of perturbation.

When the perturbation is denaturant, the REFER equation defines the Tanford β value

($\beta_T = \frac{m_f(T)}{m_{eq}(T)}$). β_T represents the relative position of the transition state with respect to a dimensionless order parameter, and provides information about the burial of solvent accessible surface area in the transition state for folding. Any perturbation which alters the free-energy of the system can be used to generate a REFER, examples include, pressure, pH, ionic strength and cosolute concentration.^{5; 6; 7; 8; 9; 10; 11; 12; 13} Mutation can also be used as a perturbation. A single point mutant together with the wild-type can be viewed as defining a two point REFER, the slope of which is traditionally denoted as the Φ -value.² In favorable cases the Φ -value can be related to the development of structure at the site being probed.^{2; 4; 14; 15; 16}

One area of controversy is whether or not transition state barriers are narrow or broad. A broad barrier is expected to lead to significant changes in the position of the transition state upon a perturbation and this has been observed in some cases.^{14; 17} In contrast, Leffler plots and the corresponding self-interaction and cross-interaction parameters (defined below) for a range of proteins were analyzed and concluded that barriers are narrow.^{6; 8; 9} A related second area of controversy is whether or not true Hammond behavior is observed in folding reactions or if apparent shifts in the position of the transition state are actually due to ground state effects.^{7; 8; 9} REFERs are a powerful tool for analyzing transition state movements. Non-linear REFERs, i.e. a value of α_x which changes as a function of x, can provide evidence for changes in the position of the transition state. Non-linearities might arise from (a) a change in the rate limiting step of the reaction, i.e. multi-state reaction mechanism, (b) a change in the reaction mechanism or (c) a change in the position of the transition state relative to the ground states, for example, Hammond behavior.⁷

Self- and cross-interaction parameters provide a simple method to analyze transition state movement.^{6; 18; 19} The self-interaction parameter, p_x is related to the movement of the transition state with a change in ΔG° caused by a single perturbation, δx :

$$p_x = \frac{\delta\alpha_x}{\delta\Delta G_x^0} = \frac{\delta^2\Delta G^\ddagger}{(\delta\Delta G_x^0)^2} \quad (2)$$

A positive p_x indicates the movement of the transition state towards the destabilized state, i.e. Hammond behavior. Non-zero self-interaction parameters give rise to non-linear REFERS. However, the shift in the transition state for protein folding is normally small and can be hard to detect with self-interaction parameters. Cross-interaction parameters, p_{xy} , are more sensitive to transition state movement and can be calculated by applying two different perturbations, denoted as x and y :²⁰

$$p_{xy} = \frac{\delta\alpha_x}{\delta\Delta G_y^0} = \frac{\delta^2\Delta G^\ddagger}{\delta\Delta G_x^0\delta\Delta G_y^0} = \frac{\delta\alpha_y}{\delta\Delta G_x^0} \quad (3)$$

We examined the barrier to the folding of NTL9, a small α - β protein. NTL9 is particularly interesting in this regard because a recent computational study, conducted with a simplified potential, suggested temperature dependent Hammond behavior.²¹ In the present work we conducted temperature and guanidinium HCl dependent studies to probe the folding barrier for NTL9 and to determine if the predicted temperature dependent Hammond behavior is indeed observed. Temperature dependent shifts in the position of the transition state are potentially very important since high temperature unfolding simulations have become a popular approach for studying folding.^{22; 23} Significant temperature dependent shifts in the transition state could call in to question the approach.

NTL9 is the N-terminal domain of ribosomal protein L9 from *Bacillus stearothermophilus* and is the simplest example of the split β - α - β motif (Figure 1). The fold is made up of a three stranded anti-parallel β -sheet sandwiched between two α -helices. The folding of NTL9 is cooperative and two-state under all conditions studied.^{24; 25; 26; 27; 28}

Results

Temperature Dependent Folding of NTL9

Fluorescence detected stopped-flow experiments were conducted between 11°C and 40°C (Figure 2). The resulting plots of $\ln k_{obs}$ vs. denaturant concentration, “chevron plots”, all had the classic V-shape expected for two-state folding. Linearity in the folding and unfolding branches of the chevron plots was observed at all temperatures studied, in particular there was no roll-over even at low concentrations of denaturant. The agreement between the ΔG° value calculated from kinetic experiments and the value determined from equilibrium studies confirm that folding is 2-state. The change in fluorescence upon the folding of NTL9 is due to a single Tyrosine located on the surface of the first helix. In principle it is formally possible that the change in fluorescence is reporting on local structure at this site. Three experimental observations rule out this possibility; first the stability calculated from k_f and k_u determined by stopped-flow fluorescence is in excellent agreement with the stability determined by CD monitored denaturation. Second, equilibrium CD and fluorescence detected stability studies give the same value of ΔG° . Thirdly, we have recently characterized a variant of NTL9 which contains the fluorescent amino acid p-cyanophenylalanine in the hydrophobic core. p-Cyanophenylalanine fluorescence can be detected independent of Tyr fluorescence and the p-cyanophenylalanine monitored kinetics agree with the tyrosine monitored kinetics.²⁹

Analysis of the data allows us to also determine m_f and m_u . m_f and m_u are believed to be related to the change in solvent accessible surface area between the unfolded and transition state and between the folded state and transition state, respectively.³⁰ m_f and m_u can be combined to yield the equilibrium m -value, m_{eq} , which is proportional to the change in solvent accessible surface area between the folded state and the denaturant induced unfolded state.^{30; 31} β_T defines the position of the transition state relative to a dimensionless order parameter. β_T also formally defines the denaturant induced interaction parameter, α_D :

$$\alpha_D = \frac{\delta\Delta G^\ddagger / \delta[\text{denaturant}]}{\delta\Delta G^0 / \delta[\text{denaturant}]} = \frac{m_f}{m_{eq}} = \beta_T \quad (4)$$

Changes in β_T (α_D) can arise because of transition state movement or because of ground state effects. The situation is illustrated in Figure 3 which is adopted from the analysis of Kiefhaber and coworkers. A particularly lucid description of this phenomenon can be found in the work of Kiefhaber.^{8; 9} The diagram schematically indicates the position of the transition state as defined by β_T . Case-b illustrates true transition state movement in the absence of ground state effects. In the case shown, the perturbation causes a shift of the transition state towards the native state. This would lead to Hammond behavior provided the perturbation destabilized, as is often the case, the folded state. The scenario illustrated in (c) also leads to an increase in β_T , but now the effect is due to the perturbation altering the unfolded state i.e. to ground state effects. Likewise an increase in β_T can result from ground state effects upon the folded state (d). The latter case is expected to be much rarer than the former. The simple diagram shows that analysis of the effects of a perturbation upon m_u and m_f provides much more information than simply examining its effect upon β_T .

There is a clear increase in m_f and a decrease in m_u for NTL9 as the temperature is increased. However, the value of m_{eq} increases with temperature since the effects on m_f and m_u are not identical (Figure 4(a)). These observations indicate transition state movement relative to the ground states as well as ground state effects involving the unfolded state. Figure 4(c) shows the change in β_T as a function of temperature. β_T increases, as the temperature is increased, indicating that the transition state potentially shifts towards the native state as the temperature is raised. Since the native state is destabilized at higher temperature, the shift represents Hammond behavior. The analysis described in the following section confirms that the shift reflects real movement of the transition state even though there are ground state effects. The data is summarized in Table-1.

Analysis of Leffler Plots and Temperature Dependent Cross-Interaction Parameter

Leffler plots, plots of $\Delta G^\ddagger$ vs. ΔG° , or $\ln k_f$ vs. $\ln K$, can be analyzed in order to detect transition state movement. The Leffler plot for the denaturant dependent data is linear, but a plot of the activation free energy, $\Delta G^\ddagger$ versus the equilibrium free energy, ΔG° constructed using the temperature dependent data is non-linear (Figure 5b). Linear Leffler plots are often observed for denaturant dependent data even when non-linearities are observed for other perturbations.^{6; 8; 9; 10} The reason for the insensitivity of denaturant dependent data to non-linearities is not understood. The non-linearity in the temperature dependent data is clear in plots of $\ln k_f$ vs. $\ln K$ and in plots of $\Delta G^\ddagger$ vs. ΔG° (Figure 5(a), 5(b)). It is not a consequence of the one data point which appears to be a slight outlier in the figures. Both curves are clearly non-linear even if that point is ignored. The nonlinearity in the temperature dependent Leffler plot appears to be very large. However, this is in part due to the compressed scale over which the data can be collected. Much larger stability changes can be probed in the denaturant dependent data.

A non-linear plot can arise from a change in the position of the transition state, a change in the reaction mechanism or a change in the rate limiting step of a multi-step reaction.^{8; 18; 19} All

of the available data indicates that the folding of NTL9 is two-state at all temperatures studied, arguing against the latter two explanations for the non-linearity. The simplest remaining explanation for the non-linear rate-equilibrium free energy relationship is movement of the transition state towards the destabilized ground state, which in this case is the native state, i.e. Hammond behavior.

Cross-interaction parameters are generally more sensitive to changes in the position of the transition state and a positive cross-interaction parameter is consistent with Hammond behavior. Thus, to test if the movement of the transition state is actually due to Hammond behavior, we calculated the denaturant-temperature cross-interaction parameter (Figure 6),

$$p_{DT} = \frac{\delta\alpha_D}{\delta\Delta G_T^0} = 0.10 \pm 0.02 \text{ mol.Kcal}^{-1} \quad (5)$$

The positive cross-interaction parameter confirms the observed Hammond behavior.

Discussion

The data analyzed here provides strong evidence that the transition state for the folding of NTL9, as judged by the Tanford β -parameter, shifts towards the native state as the temperature increases. Since the protein becomes less stable as temperature increases this provides an example of Hammond behavior, even after ground state effects are taken into account. A number of proteins are thought to undergo perturbation induced changes in the apparent position of the transition state because of a switch between consecutive transition states on a sequential pathway. NTL9 is a relatively small protein with 56 residues, and it may be that the domain is simply too small to populate the partially folded high energy intermediate that is needed for the multi-barrier scenario. Along these lines the transition state for the folding of NTL9 involves interactions in the first 39 residues thus the folding core is even smaller than the intact protein. Analysis of the dependence of m_f , m_u and the equilibrium m -value, as well as the temperature-denaturant cross-interaction parameter demonstrate that, while there are ground state effects, a shift of the transition state is observed. In particular, the coupled increase in m_f and decrease in m_u , and as a result, increase in m_{eq} , indicates the presence of both Hammond behavior and movement in the position of the unfolded state (Figure 4(b)).

The experimental studies described here are relevant to recent theoretical work reported by Pande and colleagues.²¹ Those workers used their distributed computing protocols with an implicit solvent model to characterize the temperature dependent folding of the K12M mutant of a 39 residue fragment of NTL9, denoted K12M NTL9₁₋₃₉. This fragment contains the three stranded β -sheet and first helix and thus comprises the entire split β - α - β core of NTL9. Experimental studies have shown that NTL9₁₋₃₉ adopts the same structure in isolation that it does in NTL9. Folding is two-state for both NTL9₁₋₃₉ and K12M NTL9₁₋₃₉.^{32; 33} The fragment lacks the C-terminal α -helix however, experimental work has shown that formation of this helix and its docking to the β - α - β core is not part of the rate limiting step in the folding of NTL9.³⁴ Pande et. al. choose to study the K12M mutant since it folds faster³⁵ making it more attractive for computational studies, however the folding rate is of the same magnitude as wild-type NTL9₁₋₃₉. Their calculations were approximate since the implicit solvent model used, did not change with temperature i.e. it had not been parameterized to reproduce the temperature dependence of the hydrophobic effect and solvent properties. Nevertheless, clear evidence of Hammond behavior was observed for the ensemble with detectable changes in the position of the free energy barriers and changes in their width. To the best of our knowledge that work, which was conducted completely independent of the present study, was the first to

examine potential temperature dependent Hammond behavior in a large ensemble of trajectories. The experimental data presented here provides independent verification of this theoretical prediction.

Temperature dependent changes in the position of the folding/unfolding transition state are potentially of great importance because one popular approach for computational studies of folding and unfolding is to conduct molecular dynamics simulations at very high temperature and extrapolate the results back to experimentally accessible temperatures.²² Significant shifts in the position of the transition state could be a cause for concern.²³

There have been relatively few experimental studies of the temperature dependence of protein folding and even fewer investigations of potential transition state movement as a function of temperature. Tendamistat is probably the best characterized example. In that case, the measured cross-interaction parameter, p_{DT} is 3.2×10^{-2} mol. Kcal⁻¹. The value for NTL9 is approximately three-fold larger, 0.10 mol. Kcal⁻¹. Since the cross interaction parameter is essentially reporting on the temperature dependence of β_T , it can include contributions from ground state effects. Ground state effects appear to be absent in Tendamistat but are non-zero for NTL9. Comparison of the temperature dependence of m_u is a robust method for the analysis of transition state movement since it reports on changes in the solvent accessible surface area between the native and transition state. Ground state effects are likely to be much smaller for the native state than for the unfolded state and, in any case, direct structural characterization of the native state is possible. Thus the temperature derivative of m_u suffers less from ground state effects than the temperature derivatives of m_f or β_T (i.e. the denaturant-temperature cross-interaction parameter). In the case of NTL9, careful temperature dependent NMR studies have shown that changes in native state structure are trivial over the temperature range of this study.²⁴ Comparison of the temperature derivatives of m_u for Tendamistat and NTL9 show that

transition state movement is larger for NTL9. The value of $(\frac{\partial m_u}{\partial \Delta G_f^o})$ is -0.107 M⁻¹ for NTL9 (Table 2) and -0.039 M⁻¹ for Tendamistat. The temperature dependence of m_{eq} , m_f and m_u can also be used to define a temperature-denaturant cross-interaction parameter that has been corrected for ground state effects. The value for NTL9 is still 2-fold larger than for Tendamistat (Supplementary Material). Tendamistat contains 2 disulfides which will provide covalent constraints upon the unfolded state, perhaps this is why that protein exhibits smaller ground state effects than NTL9.

Temperature dependent transition state movement has also been observed for CI-2. In that case, changes in Φ -values were used to probe the shift of the transition state. Small temperature dependent increases in the Φ -values were observed.¹⁷ In all of these studies multiple perturbations, temperature-denaturant or mutation-denaturant, i.e. cross-interaction parameters were used to detect movement in the transition state, highlighting the fact that cross-interaction parameters appear to be much more sensitive probes of such shifts than self-interaction parameters.⁸ It seems entirely reasonable that the position of the transition state of NTL9 is sensitive to temperature since it is known that formation of H-bonds and a loosely packed hydrophobic core is more important than formation of specific side chain interactions for the folding of this protein.^{27; 34} Thus, a perturbation such as temperature that globally effects these interactions, instead of local perturbations such as site specific mutations, appears to be a particularly good method for detecting transition state movement.

Materials and Methods

Materials

NTL9 was expressed as described previously.²⁸ The protein was purified with reverse-phase HPLC and the molecular weight of the protein was confirmed by MALDI-TOF mass spectroscopy.

Kinetic Measurements

Stopped-flow fluorescence measurements were conducted using an Applied Photophysics SX.18MV instrument. The change in fluorescence of Tyr25 in NTL9 was followed above 305 nm with excitation at 280 nm. Experiments were conducted in 20 mM sodium acetate, 100 mM NaCl and pH 5.4. The final protein concentration after mixing was around 30 μ M. Typically, four fluorescence traces were collected at each denaturant concentration and averaged. The average trace was fit to a single exponential function to obtain the observed rate constant (k_{obs}). The natural log of k_{obs} vs. denaturant concentration was fit to the following equation:

$$\ln(k_{obs}) = \ln(k_f^{H_2O} \exp(-m_f [\text{denaturant}]/RT) + k_u^{H_2O} \exp(m_u [\text{denaturant}]/RT)) \quad (6)$$

where $k_f^{H_2O}$ and $k_u^{H_2O}$ are the folding and unfolding rate in the absence of denaturant, respectively, m_f and m_u are the constants of proportionality for the dependence of k_f and k_u on denaturant concentration. The concentration of denaturant was determined by measuring the index of refraction.

The stability of the protein was determined from the kinetic data.

$$\Delta G^0 = -RT \ln \left(\frac{k_f}{k_u} \right) \quad (7)$$

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

K	Equilibrium constant for protein folding
k_f	Rate constant for protein folding
m_{eq}	Slope of a plot of $\ln K$ vs. denaturant concentration
m_f	Slope of a plot of $\ln(k_f)$ vs. denaturant concentration
NTL9	N-terminal domain of ribosomal protein L9 from <i>Bacillus stearothermophilus</i> , corresponding to residues 1 to 56
REFER	Rate equilibrium free energy relationship

α_x	The value of $(\frac{\partial \Delta G^\ddagger}{\partial x})/(\frac{\partial \Delta G^0}{\partial x})$ where x represents a perturbation
β_T	Tanford beta value $(\frac{m_f}{m_{eq}})$
ΔG_T^o	ΔG^o as a function of temperature
$p_{xy} = \frac{\partial \alpha_x}{\partial \Delta G_y^0} = \frac{\partial^2 \Delta G^\ddagger}{\partial \Delta G_x^0 \partial \Delta G_y^0}$	the cross-interaction parameter

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Figure 1.
A ribbon diagram of NTL9. The diagram was created using the pdb file 2HBA and the program MolMol 2K.2. The N-terminus is labeled.

Figure 2. Fluorescence monitored stopped-flow folding studies of NTL9 at different temperatures. The line is the best fit of the data to equation (6).

Figure 3.

Schematic representation of the effect of a perturbation to the unfolded (U), folded (F) and transition states (TS). (a) The initial condition prior to the perturbation. (b) Illustrates a shift in the position of the TS without any change in the position of the ground states. (c) Illustrates how ground state effects, here on the unfolded state, can cause a change in β_T which could be mistaken for Hammond behavior. (d) Ground state effects upon the native state can also lead to an apparent shift in the position of the transition state.

Figure 4. Change in m_f , m_u and m_{eq} as a function of (a) temperature, (b) ΔG_T° , where ΔG_T° is the value for ΔG° at different temperatures. (c) Changes in β_T value as a function of temperature. The line represents the linear fit to the data.

Figure 5.

Leffler plots. (a) and (b) Data collected at different temperatures. Deviation from linearity is an indication of a possible Hammond Behavior. (a) Data plotted as $\ln k_f$ vs. $\ln K$. (b) Data plotted as $\Delta G^\ddagger$ vs. ΔG° where $\Delta G^\ddagger$ is calculated using a prefactor of 10^6 . The choice of prefactor affects $\Delta G^\ddagger$, but not the slope of the plot. (c) Data collected at constant temperature, in this case 25°C, as a function of denaturant.

Figure 6.

Effect of varying ΔG° on β_T (α_D). ΔG° was varied by changing the temperature. The slope is the cross-interaction parameter, p_{DT} . A positive value of p_{DT} confirms Hammond behavior.

Table 1

Temperature dependent folding parameters for NTL9. Experiments were conducted in 20 mM NaAcetate, 100 mM NaCl, pH 5.4. The numbers after the $\pm$ sign represent the standard errors to the fit

T (°C)	k_f (sec ⁻¹)	k_u (sec ⁻¹)	m_f (kcal. Mol ⁻¹ . M ⁻¹)	m_u (kcal. mol ⁻¹ . M ⁻¹)	β_T
10.9	204.0 $\pm$ 5.0	0.15 $\pm$ 0.010	- 0.80 $\pm$ 0.010	0.58 $\pm$ 0.010	0.58
15.1	323.0 $\pm$ 9.0	0.24 $\pm$ 0.020	- 0.82 $\pm$ 0.010	0.58 $\pm$ 0.010	0.59
20.1	530.0 $\pm$ 13.0	0.50 $\pm$ 0.040	- 0.85 $\pm$ 0.010	0.55 $\pm$ 0.010	0.60
25.0	775.0 $\pm$ 20.0	0.96 $\pm$ 0.060	- 0.88 $\pm$ 0.010	0.54 $\pm$ 0.010	0.62
30.2	1140.0 $\pm$ 23.0	2.17 $\pm$ 0.100	- 0.91 $\pm$ 0.010	0.51 $\pm$ 0.010	0.64
35.0	1552.0 $\pm$ 62.0	4.17 $\pm$ 0.300	- 0.95 $\pm$ 0.020	0.50 $\pm$ 0.010	0.65
40.0	1860.0 $\pm$ 121.0	7.47 $\pm$ 1.000	- 0.95 $\pm$ 0.035	0.51 $\pm$ 0.020	0.65

p_{DT} values (uncorrected and corrected) and the variation in m_{eq} and m_f with temperature derived from the analysis of the effect of denaturant and temperature on the kinetics of NTL9

Table 2

	$\delta m_{eq}/\delta \Delta G^0_T (M^{-1})$	$\delta m_f/\delta \Delta G^0_T (M^{-1})$	$\delta m_w/\delta \Delta G^0_T (M^{-1})$	$p_{DT} = \delta a_D/\Delta G^0_T \text{ (mol. Kcal}^{-1})$	$p_{DT} = \delta a_D/\Delta G^0_T \text{ (corrected) (mol. Kcal}^{-1})$
NTL9	0.106 ± 0.0203	0.213 ± 0.0365	-0.107 ± 0.0229	0.103 ± 0.0198	0.0776 ± 0.0167