Studies of the Ankyrin Repeats of the *Drosophila melanogaster* Notch Receptor. 2. Solution Stability and Cooperativity of Unfolding[†]

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Received July 10, 2001; Revised Manuscript Received September 18, 2001

ABSTRACT: To define the boundaries of the *Drosophila* Notch ankyrin domain, examine the effects of repeat number on the folding of this domain, and examine the degree to which the modular architecture of ankyrin repeat proteins results in modular stability, we have investigated the thermodynamics of unfolding of polypeptides corresponding to different segments of the ankyrin repeats of *Drosophila* Notch. We find that a polypeptide containing the six previously identified ankyrin repeats unfolds cooperatively, but is of modest stability. However, inclusion of a putative seventh, C-terminal ankyrin sequence doubles the stability of the Notch ankyrin domain (a 1000-fold increase in the folding equilibrium constant), indicating that the seventh ankyrin repeat is an important part of the Notch ankyrin domain, and demonstrating long-range interactions among ankyrin repeats. This putative seven-repeat polypeptide also shows increases in enthalpy, denaturant dependence (*m*-value), and heat capacity of unfolding (ΔC_p) of around 50% each, suggesting that deletion of the seventh repeat results in partial unfolding of the sixth ankyrin repeat, consistent with spectroscopic and hydrodynamic data reported in the preceding paper [Zweifel, M. E., and Barrick, D. (2001) *Biochemistry 40,* 14344−14356]. A polypeptide consisting of only the five N-terminal repeats has stability similar to the six-repeat construct, demonstrating that stability is distributed asymmetrically along the ankyrin domain. These data are consistent with highly cooperative two-state folding of these ankyrin polypeptides, despite their modular architecture.

Ankyrin repeats are directly repeated 33 residue sequences that show considerable sequence variability (*1, 2*), but regular secondary and tertiary structure (*3*). Each ankyrin repeat adopts a fold consisting of two antiparallel α-helices connected by one short and one long loop (*3*−*12*). Adjacent ankyrin repeats pack against each other to form a roughly linear array of helix pairs with an extended hydrophobic core. This overall tertiary structure differs significantly from that of typical globular proteins. Whereas ankyrin repeat proteins have a very local, regular topology, consisting of regularly spaced modules that make up a nearly linear array, typical globular proteins have long-range, irregular topologies, where close contacts are made by residues very distant in primary sequence. Similar local, regular topologies are seen for other classes of repeat proteins, including armadillo repeats (*13*), leucine-rich repeats (*14*), HEAT repeats (*15*−*17*), TPR repeats (*18, 19*), β-helix proteins (*20, 21*), and WD40 repeats (*22, 23*) [for reviews, see (*24, 25*)].

The modular architecture of ankyrin repeat proteins and other repeat proteins may be reflected in the thermodynamics of unfolding of repeat proteins. Whereas the long-range interactions of typical globular proteins may lead to highly cooperative unfolding, repeat proteins may unfold in a modular, multistate reaction controlled by short-range interactions. The idea that the stability of ankyrin repeat proteins is modularly distributed is consistent with the high natural variation in the number of ankyrin repeats in different proteins (*2*). Ankyrin, the cytoskeletal protein for which the repeats are named, contains 24 repeats (*2, 26, 27*), whereas myotrophin contains only 3 repeats yet it adopts a stable fold (*28*). Further support for modular distribution of structure in ankyrin repeat proteins comes from studies of stabilities of fragments of ankyrin repeat proteins. An autonomously folding two-repeat fragment has been identified and characterized from the p16 tumor suppressor protein, which contains four ankyrin repeats (*29*). In addition, the 24 repeats of the ankyrin protein appear to be clustered into independently folded subdomains of approximately 6 repeats each (*27*).

The activity of the Notch receptor, a central component in a ubiquitous transmembrane signaling pathway (*30*−*35*), appears to be modulated through direct protein−protein interactions involving a set of ankyrin repeats in the cytosolic portion of the Notch receptor (*36*−*42*). In *Drosophila melanogaster*, six ankyrin repeats have been identified from analysis of the primary sequence of the Notch gene (*1, 2*). In the preceding paper (*43*), we have presented spectroscopic evidence that an additional C-terminal region, which partially matches the ankyrin consensus sequence, appears to be capable of folding along with the six N-terminal repeats, and to a limited degree increases the amount of structure in the six N-terminal repeats. Furthermore, we have shown that a polypeptide that contains only the five N-terminal repeats is capable of adopting the same overall secondary structural

[†] Supported by a Basil O'Connor Starter Scholar Award from the March of Dimes (5-FY98-0719), by a Young Investigator Award from the Arnold and Mabel Beckman Foundation, and by Grant GM60001 from the National Institutes of Health.

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10.1021/bi011436+ CCC: $20.00
Published on Web 11/06/2001

features as the six-repeat polypeptide. Whereas the similar structural features of the five- and six-repeat polypeptides are consistent with modular stability, the structural differences between the polypeptide containing the putative seventh repeat and the shorter polypeptides suggest that long-range interactions play an important role in determining the distribution of stability.

Here we examine the conformational stability of polypeptides from the *Drosophila* Notch ankyrin repeat region that contain different numbers of repeats. Our results provide evidence that although the five- and six-repeat constructs are stably folded, the putative seventh ankyrin repeat greatly increases the stability of the *Drosophila* Notch ankyrin domain. In addition, the seventh repeat contributes to derivative thermodynamic quantities such as enthalpy and heat capacity of folding, and to the denaturant dependence of stability. By comparison of unfolding transitions monitored by circular dichroism, fluorescence spectroscopy, and calorimetry, we find that although the six- and seven-repeat polypeptides differ significantly in stability, their unfolding transitions can be adequately described by two-state models, indicating that the ankyrin repeats of Notch unfold in a highly cooperative manner despite their modular architecture. Although removing the putative seventh repeat is strongly destabilizing, removal of the sixth repeat has almost no effect on stability. Consistent with the findings in the preceding paper (*43*), these results suggest that the sixth ankyrin repeat is folded in the seven-repeat construct, but is unfolded in the absence of the seventh C-terminal repeat.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification. Subcloning, expression, and purification of *Drosophila* Notch ankyrin repeat polypeptides were described in the preceding paper (*43*). Polypeptides studied here include the N-terminal five, six, and the putative seven ankyrin repeat sequences (Nank1−5*, Nank1−6*, and Nank1−7*),[1] and unless noted otherwise, the two internal cysteines have been replaced with serines and the N-terminal cysteine has been omitted. We use asterisks (e.g., Nank1−5*) to indicate that cysteines have been replaced with serine. These serine substitutions were found to prevent a slow loss of material during equilibrium ultracentrifugation (*43*).

Urea-Induced Unfolding Transitions. Both CD and fluorescence-detected unfolding transitions were acquired on an Aviv 62A DS spectropolarimeter (Aviv Associates, Inc., Lakewood, NJ). Circular dichroism was detected at 222 nm. Fluorescence was detected by exciting at 280 nm and recording emission using a perpendicular photomultiplier tube behind a 320 nm cutoff filter. Urea-induced unfolding transitions were generated using a computer-controlled Hamilton Microlab 500 titrator with two 500 μL syringes (*44*, *45*). Titrations began with 2.0 mL of protein in 150 mM NaCl, 25 mM Tris·HCl, pH 8.0, in the cuvette. To generate the titration, a portion of the starting sample was removed and replaced with an equal volume of protein in the same buffer and salt, containing approximately 7.5 M urea. After 300 s to allow for mixing, thermal, and chemical equilibration, CD and fluorescence signals were recorded, and the procedure was repeated. In manual mixing experiments, we found that this 300 s period was sufficient for equilibration at different urea concentrations, including those of the transition zone, where equilibration is likely to be slowest. Protein concentrations were typically 2−5 μM in a cuvette of 1 cm path length, although it was found that identical curves were obtained over a range of 1−10 μM. To ensure reversibility in urea-induced unfolding transitions, unfolded protein in different urea concentrations was diluted into buffer under folding conditions, and the final CD signal was compared to the value obtained for folded protein in the same final conditions. In these tests, the same value was obtained whether the protein had initially been unfolded.

Thermodynamic unfolding parameters were evaluated using the linear extrapolation method (*46*, *47*), where the free energy of unfolding varies linearly with urea concentration through the equation:

$$\Delta G^\circ = \Delta G^\circ(\mathrm{H_2O}) - m[\mathrm{urea}] \quad (1)$$

This equation was related to the concentration of native (N) and denatured (D) protein through the exponential form of eq 1 in which ΔG° is substituted by $-RT \ln K_u$ (where K_u represents the unfolding equilibrium constant, [D]/[N]):

$$K_u = K_u(\mathrm{H_2O}) \exp^{(m[\mathrm{urea}]/RT)} \quad (2)$$

This expression is then related to the observed spectroscopic signal (Y_{obs}, either fluorescence or CD), by using the definition of the equilibrium constant in eq 2 to give a population-weighted average of the spectroscopic signals of the native and denatured proteins (Y_N and Y_D, respectively):

$$Y_{obs} = f_N Y_N + f_D Y_D = \left(\frac{1}{1+K_u}\right) Y_N + \left(\frac{K_u}{1+K_u}\right) Y_D \quad (3)$$

For data analysis, Y_N and Y_D were allowed to vary linearly with urea concentration. Equation 3 was then fitted to urea unfolding transitions using nonlinear least-squares analysis with the program Kaleidegraph 3.0 (Synergy Software, Reading, PA) to determine $\Delta G^\circ(\mathrm{H_2O})$ and m, along with the four baseline parameters.

Determination of ΔC_p from Urea-Induced Unfolding. To estimate ΔC_p from urea-induced unfolding transitions, $\Delta G^\circ(\mathrm{H_2O})$ values at different temperatures were fitted by an integrated form of the Gibbs−Helmholtz equation:

$$\Delta G^\circ = \Delta H^\circ(T_m) + \Delta C_p(T - T_m) - T\left[\Delta S^\circ(T_m) + \Delta C_p \ln\left(\frac{T}{T_m}\right)\right] = \Delta H^\circ(T_m)\left(1 - \frac{T}{T_m}\right) + T\Delta C_p\left[1 - \frac{T_m}{T} - \ln\left(\frac{T}{T_m}\right)\right] \quad (4)$$

[1] Abbreviations: Nank1−5*, Nank1−6*, and Nank1−7*, Notch ankyrin repeat polypeptides containing five, six, and seven repeats, and with the two internal cysteines replaced with serines; Nank1−6, Notch ankyrin repeat polypeptide containing six ankyrin repeats, but containing two internal and one N-terminal cysteine; CD, circular dichroism; DSC, differential scanning calorimetry; $\Delta G^\circ(\mathrm{H_2O})$, standard-state Gibbs free energy of unfolding in the absence of denaturant; T_m, temperature midpoint for unfolding; ΔH°, ΔH°_{vH}, and ΔH°_{cal}, standard-state, van't Hoff, and calorimetrically determined enthalpies of unfolding; ΔS°, standard-state entropy of unfolding; ΔC_p, difference in heat capacity between native and denatured protein.

using nonlinear least-squares analysis. In eq 4, T_m is the midpoint of the unfolding transition [$\Delta G°(H_2O) = 0$], and $\Delta H°(T_m)$ and $\Delta S°(T_m)$ are the unfolding enthalpy and entropy at T_m, respectively. Equation 4 treats ΔC_p as temperature-independent. The second equality results from the fact that $\Delta H°(T_m) = T_m \Delta S°(T_m)$. Equation 4 contains three independent parameters that require fitting: ΔC_p, $\Delta H°(T_m)$, and T_m.

In the course of analyzing our data, we found that for urea-induced unfolding transitions measured at high temperatures (where native baselines are poorly defined), fitted native baseline parameters are often inconsistent with those from transitions measured at lower temperatures. Thus, we used native baseline data from urea melts at and below 30 °C (Nank1−7*) and 20 °C (Nank1−6*) to obtain a linear coefficient describing the change in CD signal with urea concentration. We then imposed this slope in fitting the high-temperature transitions. Constraining the fit in this way had no perceptible effect on the quality of the fit, although it did produce increases in $\Delta G°(H_2O)$ of up to 0.5 kcal·mol^{-1} for unfolding transitions measured at the highest temperatures. We consider this constrained fitting procedure to be an improvement, since it makes use of baselines that are physically reasonable. Since determination of ΔC_p is very sensitive to $\Delta G°(H_2O)$ values measured at high temperatures, where native baseline parameters are poorly defined (our values of ΔC_p decrease by as much as 0.5 kcal·mol^{-1}·K^{-1} as a result of constraining the native baselines), attention should be given to native baseline parameters when determining ΔC_p using this method.

Thermally Induced Unfolding Transitions. Thermally induced unfolding transitions of Nank1−7* and Nank1−6* were monitored by CD at 222 nm. Samples contained 150 mM NaCl, 25 mM Tris·HCl, pH 8; in addition, most samples contained 1 M urea. Protein concentrations were typically 25 μM in a cuvette of 0.1 cm path length. Thermal unfolding scans were generated by raising the sample temperature in 1 °C steps, with 60 s minimum equilibration time prior to measurement of CD. To assess reversibility, refolding transitions were obtained immediately after unfolding, using the same temperature decrements and equilibration time as for the unfolding transitions.

Enthalpies and entropies of unfolding were estimated using nonlinear least-squares analysis to fit a variety of equations to the thermal unfolding curves. The simplest equation used to describe the data is a variant of eq 3, in which the unfolding equilibrium constant varies as a function of temperature instead of urea concentration:

$$K_u = \exp^{(-1/RT)(\Delta H° - T\Delta S°)} \qquad (5)$$

Equation 5 treats $\Delta S°$ and $\Delta H°$ as temperature independent, which is valid only for reactions that do not result in heat capacity change. When eq 5 is substituted into eq 3, there are six unknown parameters that require fitting: $\Delta H°$ and $\Delta S°$, the quantities of interest, and the four baseline parameters (a linear variation in CD signal with temperature was observed for both the low- and high-temperature baselines). Although the variations in $\Delta H°$ and $\Delta S°$ through the narrow transition region (where these parameters are determined) are expected to be modest, the variation of $\Delta G°$ as a function of T shows ΔC_p to be nonzero, and thus $\Delta H°$ and $\Delta S°$ must depend on temperature. To account for this temperature dependence, eq 4 was related to the unfolding equilibrium constant and rearranged to yield

$$K_u = e^{(-1/RT)\{\Delta H°(T_m)(1-(T/T_m))+T\Delta C_p(1-(T_m/T)-\ln(T/T_m))\}} \qquad (6)$$

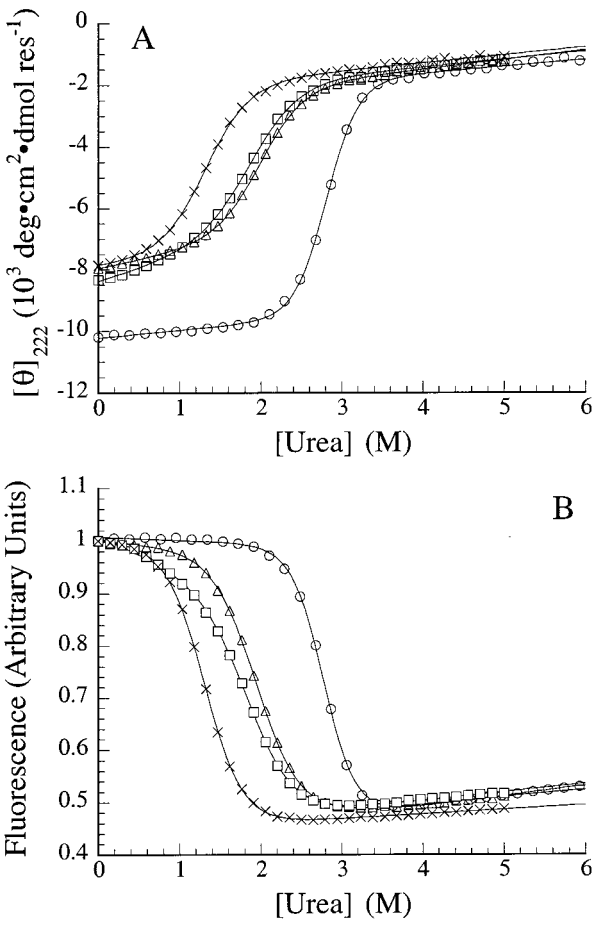

FIGURE 1: Urea-induced unfolding transitions of ankyrin repeat polypeptides from *Drosophila* Notch. Unfolding transitions of Nank1−7* (○), Nank1−6* (△), Nank1−6 (×), and Nank1−5* (□), monitored by (A) circular dichroism spectroscopy at 222 nm, and by (B) fluorescence spectroscopy. Protein concentrations were 2.5−5 μM. Conditions: 25 mM Tris, 150 mM NaCl, pH 8.0, 5 °C.

Fitting of equations based on eqs 5 and 6 was carried out using Nonlin for Macintosh (*48*). Accurate determination of thermodynamic parameters based on fitting eqs 5 and 6 to thermal unfolding transitions requires some care, since determination of these parameters requires determination of the first and second derivatives of the unfolding curves, and because only a limited portion of the denatured baseline could be used to ensure reversibility. In fitting the simple van't Hoff equilibrium expression to the data (eq 5), care was taken to make sure that fitted values of $\Delta H°$ and $\Delta S°$ did not depend on initial guesses in the fitting procedure. It was found that a wide range of $\Delta H°$ and $\Delta S°$ values converged on the same final values, but that the difference in initial guesses for $\Delta H°$ and $\Delta S°$ had to be such that $\Delta G°$ was near zero at the transition temperature, or convergence could not be achieved. Fitting eq 6 directly to thermal unfolding transitions introduces an additional parameter, ΔC_p, which is related to the second derivative of the unfolding transition. Since estimation of the second derivative of a narrow

Table 1: Urea-Induced Unfolding Parameters for Ankyrin Repeat Polypeptides from the *Drosophila* Notch Protein Monitored by CD and Fluorescence[a]

	$\Delta G°_{H_2O}$ (kcal·mol^{-1})		m (kcal·mol^{-1}·M^{-1})	
	CD	fluorescence	CD	fluorescence
Nank1−7*	8.03 ± 0.09 (n = 6)	7.99 ± 0.04 (n = 4)	2.79 ± 0.02 (n = 6)	2.80 ± 0.01 (n = 4)
Nank1−6*	4.14 ± 0.05 (n = 4)	4.04 ± 0.01 (n = 3)	2.06 ± 0.02 (n = 4)	2.05 ± 0.01 (n = 3)
Nank1−6	2.74 ± 0.04 (n = 3)	3.00 ± 0.07 (n = 3)	2.05 ± 0.02 (n = 3)	2.29 ± 0.05 (n = 3)
Nank1−5*	3.55 ± 0.25 (n = 3)	3.70 ± 0.16 (n = 3)	1.85 ± 0.10 (n = 3)	1.97 ± 0.04 (n = 3)

[a] Parameters estimated by fitting the linear free energy equation (eq 1) to the unfolding transitions. Reported values are the mean of the number of independent unfolding transitions analyzed (n, as given in parentheses). Uncertainties are standard errors on the mean, that is, the standard deviation divided by $n^{1/2}$. Conditions: 25 mM Tris·HCl, 150 mM NaCl, pH 8, 4 °C.

unfolding curve is expected to be subject to a high degree of uncertainty, we have used estimates of ΔC_p from urea-induced unfolding (see above) in eq 6, in addition to fitting ΔC_p as an independent parameter.

Differential Scanning Calorimetry. Notch ankyrin polypeptides were dialyzed extensively into 25 mM Tris·HCl, 150 mM NaCl, 1 M urea, pH 8. Polypeptides were then diluted into dialysis buffer to approximately 20 μM. Excess heat capacity (over dialysis buffer) was measured at a scan rate of 0.5 K·min^{-1} on a Microcal model VP-DSC differential scanning microcalorimeter (Northampton, MA). Native and denatured baselines were fitted either using linear functions with different intercepts for the pre- and post-transition regions but identical slopes (maintaining a temperature-independent ΔC_p value), or using separate slopes for each baseline. The progress baseline routine of the Microcal Origin software package was then used to generate baselines through the transition region.

RESULTS

Urea Denaturation of the Drosophila Notch Ankyrin Repeats. To assess the role of the sixth and putative seventh repeats in stabilizing the ankyrin domain of the *Drosophila* Notch protein, and to determine whether individual ankyrin repeats contribute to stability in a modular way, we have performed urea-induced denaturation studies of three different Notch polypeptides: Nank1−6*, which contains the six ankyrin repeats identified in the literature (*2*); Nank1−7*, which contains an additional putative C-terminal repeat; and Nank1−5*, which contains only the five N-terminal repeats [see Table 1 of (*43*)]. If individual repeats are modular in stability, that is, if their stabilities are independent of each other, then shallow multistate unfolding transitions should be observed, which should be insensitive to deletion of terminal repeats. If instead there is a high degree of stabilizing interaction among repeats, cooperative two-state unfolding transitions should result, and should be highly sensitive to deletion of terminal repeats.

Urea-induced unfolding transitions of *Drosophila* Notch ankyrin repeat polypeptides have cooperative, sigmoidal shapes when monitored by CD spectroscopy at 222 nm, which measures α-helix content, and by tryptophan fluorescence, which measures the polarity of the tryptophan in repeat 5 (Figure 1). Both CD and fluorescence unfolding curves can be well-fitted using the linear extrapolation method (eqs 1−3) to estimate stability as a function of urea concentration (solid lines, Figure 1). Furthermore, we find that for a given ankyrin repeat polypeptide, similar thermodynamic parameters [$\Delta G°(H_2O)$, m] are obtained from CD and from fluorescence-monitored transitions (Table 1). This is especially true for Nank1−7* and Nank1−6*, where the thermodynamic parameters are the same within error; for instance, estimated $\Delta G°(H_2O)$ values for Nank1−7* are 8.03 ± 0.09 and 7.99 ± 0.04 kcal·mol^{-1} from CD and fluorescence transitions, respectively, and estimated m-values are 2.79 ± 0.02 and 2.80 ± 0.01 kcal·mol^{-1}·M^{-1} from CD and fluorescence transitions, respectively. The slight discrepancies between $\Delta G°(H_2O)$ measured by CD versus fluorescence for Nank1−6 and Nank1−5* may reflect the greater uncertainty in fitted parameters resulting from poorly defined native baselines for these two polypeptides.

Although the same thermodynamic parameters are obtained by CD- and fluorescence-detected unfolding transitions for each Notch ankyrin polypeptide, different ankyrin polypeptides give very different unfolding curves. In particular, the ankyrin repeat polypeptide that contains the putative seventh, C-terminal repeat shows a greatly increased stability compared to polypeptides containing six and five repeats. Nank1−7* remains in a native conformation until denaturant concentrations exceed those that result in complete unfolding of Nank1−6* and Nank1−5*. The unfolding midpoint of Nank1−7* occurs around 2.8 M urea, compared to 2.0 M urea for Nank1−6* (Figure 1). Using the linear extrapolation method, the free energy of unfolding, extrapolated to zero denaturant [$\Delta G°(H_2O)$], is around 8.0 kcal·mol^{-1} for Nank1−7* (detected by CD and fluorescence, respectively), compared to around 4.1 kcal·mol^{-1} for Nank1−6* (Table 1). This large increase in $\Delta G°(H_2O)$ demonstrates that the putative seventh, C-terminal ankyrin repeat greatly stabilizes the entire ankyrin domain of *Drosophila* Notch, and is an integral part of the domain in terms of both structure and stability.

In addition to an increase in $\Delta G°(H_2O)$, the steepness of the unfolding transition is greater for Nank1−7* than for Nank1−6*. This increase in steepness is reflected in the fitted m-values: Nank1−7* has an m-value of 2.79 kcal·mol^{-1}·M^{-1}, compared to 2.05 kcal·mol^{-1}·M^{-1} for Nank1−6* (Table 1). As m-values are often considered to be measures of the change in the exposed surface area on unfolding (*47*, *49*, *50*), these results suggest a significantly larger change in the exposed surface area on unfolding of Nank1−7* than Nank1−6*.

In contrast to the large effect on urea-induced unfolding that results from deletion of the putative seventh ankyrin repeat, further deletion of the sixth ankyrin repeat has only minor effects on the urea-induced unfolding curves (Figure 1). $\Delta G°(H_2O)$ is decreased by a modest 0.5 kcal·mol^{-1} for Nank1−5* compared with Nank1−6* (Table 1). Similarly,

Table 2: Thermodynamic Parameters for Unfolding of the Ankyrin Repeat Polypeptides of the Notch Receptor from Urea-Induced Unfolding Transitions at Different Temperatures[a]

	0 M urea			1 M urea		
	ΔC_p (kcal·mol^{-1}·K^{-1})	$\Delta H°_{vH}$ (kcal·mol^{-1})	T_m (°C)	ΔC_p (kcal·mol^{-1}·K^{-1})	$\Delta H°_{vH}$ (kcal·mol^{-1})	T_m (°C)
Nank1−7*	4.14 (*4.14, 4.14*)	149.1 (*148.7, 149.5*)	45.1 (*45.1, 45.1*)	3.45 (3.47, 3.44)	108.1 (108.5, 107.9)	39.5 (39.5, 39.4)
Nank1−6*	2.21 (*2.15, 2.28*)	77.0 (*76.1, 77.9*)	39.1 (*38.9, 39.3*)	1.71 (1.73, 1.68)	47.3 (47.7, 46.9)	32.0 (31.7, 32.3)

[a] Values are obtained from extrapolating the free energy of urea-induced unfolding at different temperatures to either 0 or 1 M urea, followed by a fitting of the extrapolated free energy using eq 4. Average values are presented from fits of two independent families of unfolding curves (individual values are indicated in parentheses). Conditions: 25 mM Tris·HCl, 150 mM NaCl, pH 8.

the *m*-value decreases by 0.2 kcal·mol^{-1}·M^{-1} for Nank1−5* compared with Nank1−6*, close to the uncertainty of this fitted parameter.

We also used urea-induced unfolding to assess the effect of cysteine replacement on stability. Comparison of Nank1−6* with Nank1−6 (a polypeptide containing all three cysteines) shows that replacement of cysteines increases the stability of the ankyrin domain (Figure 1). This stabilization results from a shift in the unfolding midpoint to higher concentrations without changing the overall shape of the unfolding curve. This shift results in an increase in $\Delta G°(H_2O)$ of around 1.2 kcal·mol^{-1} for the cysteine-free protein, without significant change in the *m*-value (Table 1). The increase in stability suggests that replacement of the cysteines does not disrupt structure.

Heat Capacity Change on Unfolding of Ankyrin Repeat Polypeptides from the Drosophila Notch Protein. The urea-induced unfolding studies indicate that addition of the putative seventh repeat significantly increases the *m*-value, and thus the exposed surface area upon unfolding (compare Nank1−7* with Nank1−6*; Table 1). We have also examined the change in exposed surface area upon unfolding by measuring the heat capacity of unfolding (ΔC_p). We have estimated ΔC_p values for Nank1−7* and Nank1−6* by measuring urea-induced unfolding curves at different temperatures. ΔC_p is obtained by fitting eq 4 to a plot of the resulting $\Delta G°(H_2O)$ values as a function of temperature (*47, 51, 52*).

Urea-induced unfolding curves of Nank1−7* and Nank1−6* shift to lower urea concentrations as the temperature is raised (Figure 2A,B, respectively). This shift is gradual at low temperatures, and then becomes more pronounced at high temperatures. Unfolding free energies obtained from the linear extrapolation method show marked downward curvature when plotted as a function of temperature (Figure 2C), indicating that the denatured states of both Nank1−7* and Nank1−6* have larger heat capacities than the native forms (i.e., ΔC_p for unfolding >0), as is commonly observed for globular proteins. From examination of the primary data, the magnitude of the curvature appears to be larger for Nank1−7* than for Nank1−6*, indicating a larger ΔC_p for the longer polypeptide. This is confirmed using nonlinear least-squares analysis to fit eq 4 to the free energy curves in Figure 2C: for Nank1−7*, a ΔC_p of 4.1 kcal·mol^{-1}·K^{-1} is obtained, whereas for Nank1−6* a value of 2.2 kcal·mol^{-1}·K^{-1} is obtained (Table 2).[2] This result suggests that Nank1−7* buries significantly more nonpolar surface area in folding than does Nank1−6*.

Thermal Unfolding of the Drosophila Ankyrin Repeats. Like *m*-values and ΔC_p, reaction enthalpy and entropy provide a measure of the sensitivity of populations of molecules to perturbations and thus provide clues as to the molecular origins of population differences. To further understand the origins of the stability enhancement of Nank1−7* compared with Nank1−6*, we have used thermal denaturation as a means to estimate the apparent or van't Hoff unfolding enthalpies ($\Delta H°_{vH}$), and, by difference, the entropy of unfolding ($\Delta S°$) of these polypeptides.

To measure $\Delta H°$ and $\Delta S°$, thermal unfolding transitions must be reversible. Reversibility can be tested by cooling the sample upon completion of thermal denaturation; if the reaction is reversible, the refolding (cooling) and unfolding (heating) curves should match, and the original native signal should be recovered at the end of the cooling transition. For Nank1−7*, only a small amount of native CD signal is recovered following thermal denaturation, demonstrating that under these conditions, thermal denaturation is not reversible (Figure 3A). Furthermore, the unfolding transition for Nank1−7* shows a peculiar hump at around 47 °C, and at higher temperatures, ellipticity becomes more negative. However, by adding 1 M urea, this hump can be suppressed, and almost all of the native CD signal can be recovered by cooling the sample, especially if the sample is kept below 52 °C. Although for Nank1−6* this hump was not seen in the absence of urea, and the folding transition was nearly completely reversed upon cooling (not shown), we have included 1 M urea in all thermal denaturations to allow direct comparison of reversible unfolding enthalpies and entropies of all Notch ankyrin polypeptides.

The reversible thermal denaturation transitions of Nank1−7* and Nank1−6* show sigmoidal unfolding transitions, and the midpoint of unfolding of Nank1−7* is significantly higher (40 °C) than that of Nank1−6* (∼30 °C, Figure 3B), consistent with the increase in stability

[2] These two values represent the average from two separate sets of urea melts that were generated on different months. $\Delta G°(H_2O)$ values from these separate data sets were fitted separately by eq 4 (indicated with the different symbols and fitted curves in Figure 2C) to yield two independent estimates of ΔC_p (Table 2). These values differ from their mean by less than 1 and 3% for Nank1−7* and Nank1−6*, respectively. These variations are significantly smaller (by around 70-fold) than the difference in ΔC_p values for Nank1−7* compared with Nank1−6*, indicating that the ΔC_p difference between these two polypeptides is statistically significant.

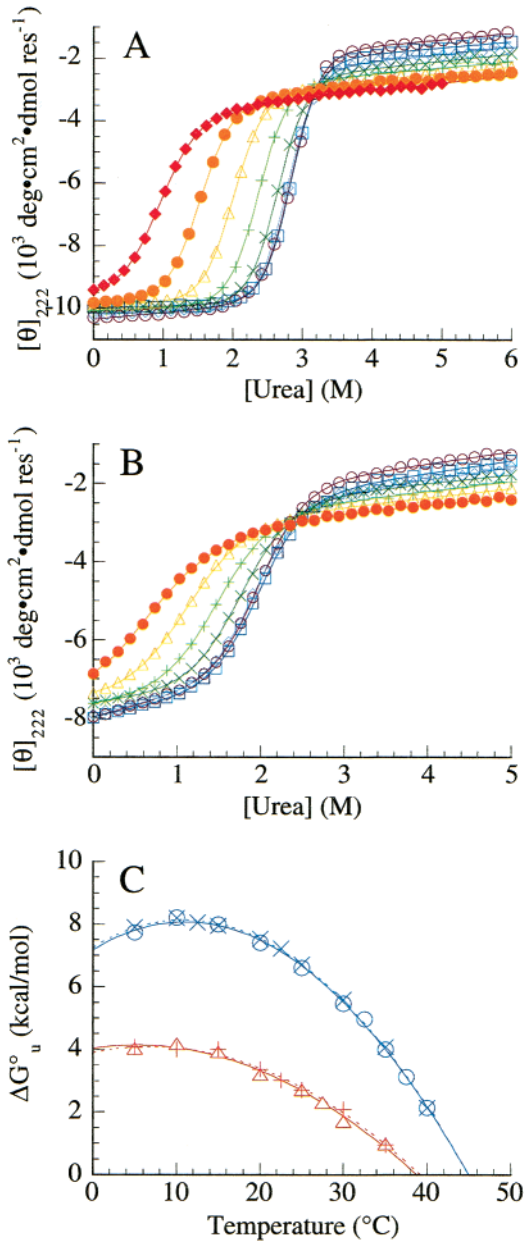

FIGURE 2: Urea-induced unfolding of the ankyrin repeat polypeptides from the *Drosophila* Notch protein as a function of temperature. Unfolding of (A) Nank1−7* and (B) Nank1−6* at 5 (○), 10 (□), 15 (◇), 20 (×), 25 (+), 30 (△), 35 (●), and 40 °C (◆; Nank1−7* only). (C) $\Delta G°(H_2O)$ versus temperature for unfolding of Nank1−7* (○, ×) and Nank1−6* (△, +). For each protein, the different symbols indicate separate data sets; each data set was fitted independently by eq 4, resulting in the two different lines (solid and dashed) through the data sets for each polypeptide. Conditions as in Figure 1.

observed for Nank1−7* from urea-induced unfolding experiments. In addition, the thermal unfolding transition of Nank1−7* is significantly steeper than that of Nank1−6*, indicating a larger enthalpy of unfolding for Nank1−7*. Using nonlinear least-squares to fit the simple van't Hoff thermal unfolding model to the data (eqs 3 and 5; see Experimental Procedures), $\Delta H°_{vH}$ values of 92 and 43 kcal·mol^{-1} were obtained for Nank1−7* and Nank1−6*, respectively (Table 3, "method A"). Although this model gives a reasonable fit to the data, it treats $\Delta H°$ and $\Delta S°$ as temperature-independent quantities. To allow for a temperature dependence in $\Delta H°$ and $\Delta S°$, eq 6 was fitted to the data. Since we expected uncertainties in estimates of ΔC_p from single thermal unfolding curves to be large, we fixed ΔC_p at values obtained through analysis of urea unfolding curves at various temperatures (Table 3, "method B").[3] When ΔC_p is treated as a constant in eq 6, there are six adjustable parameters (including the four baseline parameters), the same number of parameters as in eq 5. Thus, the decrease in the square root of the variance of the fit using method B (by around 50% for Nank1−7*, not shown) demonstrates that although the transitions are sensitive to population differences over a narrow temperature range, the effect of temperature on $\Delta H°$ and $\Delta S°$ is significant through the thermal transitions. When ΔC_p is treated as an adjustable parameter in the fitting procedure (Table 3, "method C"), fitted values of ΔC_p are surprisingly close to values obtained by analysis of urea induced unfolding curves at different temperatures (Table 2, 1 M urea), differing by around 0.6 and 0.1 kcal·mol^{-1}·K^{-1}

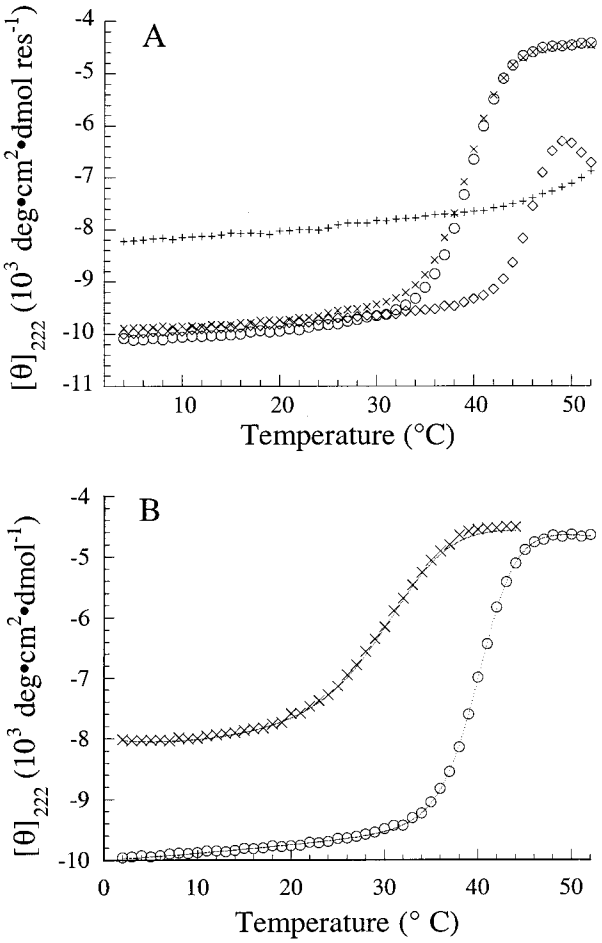

FIGURE 3: Thermal unfolding of the ankyrin repeats of *Drosophila* Notch. (A) Unfolding (heating) transitions (◇) and subsequent refolding (cooling) transitions (+) for Nank1−7* in the absence of urea, and unfolding (○) and refolding transitions (×) of Nank1−7* in the presence of 1 M urea. (B) Comparison of thermal unfolding transitions for Nank1−7* (○) and Nank1−6* (×) in 1 M urea. Solid lines show the best-fitted curves to the unfolding data, using eq 6 with heat capacities fixed at values determined in 1 M urea (Table 2). Conditions: 150 mM NaCl, 25 mM Tris·HCl, pH 8, 1 M urea in panel B and as indicated in panel A.

[3] For this procedure, ΔC_p values were evaluated by extrapolation of unfolding free energies to 1 M urea, to match conditions during thermal unfolding.

Table 3: Unfolding Enthalpies for Ankyrin Repeat Polypeptides from *Drosophila* Notch Monitored by Circular Dichroism Spectroscopy and Differential Scanning Calorimetry[a]

	CD spectroscopy				DSC
	method A	method B	method C		
	ΔH°_{vH} (kcal·mol^{-1})	ΔH°_{vH} (kcal·mol^{-1})	ΔH°_{vH} (kcal·mol^{-1})	ΔC_p (kcal·mol^{-1}·K^{-1})	ΔH°_{cal} (kcal·mol^{-1})
Nank1−7*	92 ± 2.1	96 ± 1.4	98 ± 2.1	4.0 ± 0.3	97
Nank1−6*	43 ± 0.7	45 ± 0.6	45.5 ± 0.7	1.8 ± 0.1	47−63

[a] Uncertainties in thermodynamic parameters determined by CD represent standard deviations on the mean for three (Nank1-7*) and four (Nank1−6*) separate thermal denaturation transitions. In method A, eq 5 is fitted to thermal unfolding transitions. In method B, eq 6 is fitted to thermal unfolding transitions holding ΔC_p constant at values determined from analysis of urea-induced unfolding transitions at different temperatures (3.45 and 1.71 kcal·mol^{-1}·K^{-1} for Nank1−7* and Nank1−6*, respectively, each in 1 M urea). In method C, ΔC_p is allowed to vary during the fitting of eq 6 to the data. Conditions: 150 mM NaCl, 25 mM Tris·HCl, 1 M urea, pH 8.

for Nank1−7* and Nank1−6*. Although estimates of ΔC_p obtained through these two procedures are similar, the estimates from the urea-induced unfolding transitions are probably of higher accuracy since they are obtained from analysis of equilibrium over a much broader temperature range than individual thermal unfolding transitions.

Regardless of the method of analysis of the thermal unfolding transitions, the different methods result in similar unfolding enthalpies, ranging from 92 to 98 kcal·mol^{-1} for Nank1−7*, and from 43 to 45.5 kcal·mol^{-1} for Nank1−6* (Table 3). The calculated unfolding enthalpy for Nank1−7* at its T_m is significantly larger than for Nank1−6* at its T_m, consistent with the steeper thermal unfolding transition seen for Nank1−7* (Figure 3). To directly compare the unfolding enthalpies of Nank1−7* and Nank1−6*, they must be extrapolated to a common temperature (T'). This can be done using the expression:

$$\Delta H^\circ_{T'} = \Delta H^\circ_{T_m} + \Delta C_p(T' - T_m) \quad (7)$$

Using values of ΔC_p determined from the temperature dependence of the free energy of unfolding of Nank1−7* and Nank1−6* in 1 M urea (Table 2), and extrapolating to a T' of 36 °C (midway between the T_m's of Nank1−7* and Nank1−6* in 1 M urea, minimizing extrapolation error), values of ΔH° of 84 and 52 kcal/mol are obtained for Nank1−7* and Nank1−6*, respectively.[4] Because ΔH° values for Nank1−7* and Nank1−6* differ significantly more than corresponding ΔG° values, there must be a large compensatory difference in ΔS° values between Nank1−7* and Nank1−6*. For Nank1−7*, ΔS° is 0.31 kcal·mol^{-1}·K^{-1} at its T_m, significantly larger than the corresponding value of 0.15 kcal·mol^{-1}·K^{-1} for Nank1−6* at its T_m.[5] Extrapolating to the same common temperature ($T' = 36$ °C) using the expression

$$\Delta S^\circ_{T'} = \Delta S^\circ_{T_m} + \Delta C_p \ln\left(\frac{T'}{T_m}\right) \quad (8)$$

values of ΔS° of 0.27 and 0.17 kcal·mol^{-1}·K^{-1} are obtained for Nank1−7* and Nank1−6*, respectively.

Differential Scanning Calorimetry of the Drosophila Ankyrin Repeats. One possible interpretation of the large decreases in the *m*-value and van't Hoff enthalpy of unfolding of Nank1−7* compared to Nank1−6* is that the Notch ankyrin repeat polypeptides may adopt a significant population of intermediate conformations in the thermal and urea transition regions, despite the coincidence of CD and fluorescence signals in monitoring these transitions. Such deviation from two-state behavior would decrease both of these parameters, especially if the intermediates had spectroscopic properties that were midway between those of the folded and fully unfolded proteins. To test for intermediates, and to further examine whether stability is modular in these ankyrin repeats, we measured the heat associated with the thermal unfolding of Nank1−6* and Nank1−7* by differential scanning calorimetry (DSC). Comparing direct, calorimetrically measured heats of unfolding (ΔH°_{cal}) with those determined using a two-state model (ΔH°_{vH}) tests the validity of the two-state model, namely, that only native and denatured states are highly populated, and that intermediate states are absent. If unfolding proceeds without intermediates, ΔH°_{cal} will have the same numerical value as ΔH°_{vH} ($\Delta H^\circ_{vH}/\Delta H^\circ_{cal} = 1$), whereas if intermediates are populated, ΔH°_{cal} is likely to exceed ΔH°_{vH} ($\Delta H^\circ_{vH}/\Delta H^\circ_{cal} < 1$).

As with thermal unfolding transitions monitored by CD, the calorimetric transition of Nank1−7* in 1 M urea appears to be significantly sharper than that of Nank1−6* (Figure 4). Continued scanning to higher temperatures produced a nonlinear baseline (not shown), perhaps representing a heat of reaction for an irreversible process. For Nank1−7*, the calorimetric enthalpy of the major unfolding transition (obtained as the area between the DSC scan and the fitted baseline, normalized to the number of moles of polypeptide) is very similar to values obtained using a two-state model (Table 3). Using the two-state enthalpy estimated using method B ($\Delta H^\circ_{vH} = 96$ kcal·mol^{-1}, Table 3), a ΔH°_{vH} to ΔH°_{cal} ratio of 0.99 is obtained, which is consistent with two-state unfolding of Nank1−7*.

For Nank1−6*, the calorimetric enthalpy of the major unfolding transition is difficult to quantify because the transition is rather broad, and the baselines are not well-defined. With the requirement that the native and denatured baselines have the same slope (thus maintaining a constant ΔC_p value), a calorimetric enthalpy of 63 kcal·mol^{-1} is estimated. If, instead, separate baselines are fitted to the low- and high-temperature regions of the Nank1−6* DSC scan (which appear to fit better than those that maintain a constant ΔC_p), an enthalpy of 47 kcal·mol^{-1} is obtained. Enthalpy estimates from two-state models are close to this lower value, and again using method B to estimate a two-state unfolding

[4] ΔH°_m values were taken from method B, where eq 6 was fitted to the CD-detected thermal unfolding data, holding ΔC_p constant at values determined from analysis of urea-induced unfolding transitions.

[5] At T_m, where $\Delta G^\circ = 0$, $\Delta H^\circ = T_m \Delta S^\circ$, or $\Delta S^\circ = \Delta H^\circ/T_m$.

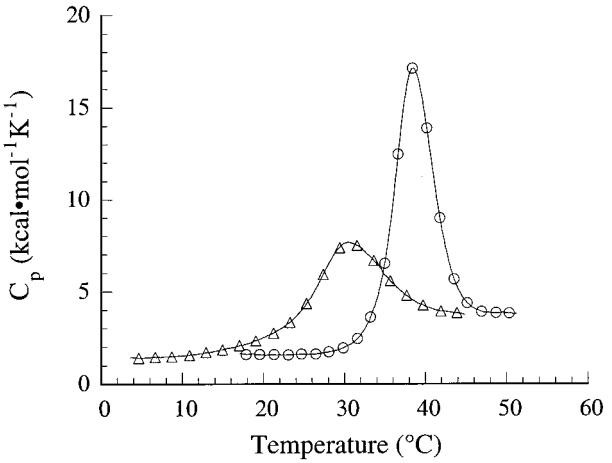

FIGURE 4: Differential scanning calorimetry of ankyrin repeat polypeptides from the *Drosophila* Notch protein. Excess heat capacity of Nank1−7* (○) and Nank1−6* (△), each at 0.5 mg/mL, recorded at a scan rate of 0.5 K•min^{-1} in 1 M urea. A parallel scan of dialysis buffer was subtracted from the data. Curves are normalized to the number of moles of polypeptide to yield molar excess heat capacity. Conditions: 150 mM NaCl, 25 mM Tris•HCl, 1 M urea, pH 8.

enthalpy yields a $\Delta H°_{vH}$ to $\Delta H°_{cal}$ ratio of 0.96. However, with the larger estimate of $\Delta H°_{cal}$, this ratio becomes 0.71. Thus, for Nank1−6*, the calorimetry data are somewhat inconclusive: while they are consistent with two-state unfolding, they can equally be interpreted as an indication of multistate unfolding.

DISCUSSION

Cooperative, Two-State Unfolding of Notch Ankyrin Repeat Polypeptides. Ankyrin repeat proteins differ from typical globular proteins in that the tertiary structure of ankyrin domains consists of a regular, linear array of modules that make only short-range interactions. This modular architecture raises the possibility that the stability of ankyrin domains may be modular as well; that is, to some degree, the individual modules may be thermodynamically independent of one another, and may unfold independently of their neighboring repeats. As the ankyrin repeats of the Notch receptor are involved in a number of different binding interactions that regulate signaling, allostery must play an important role in function. Modular stability would have important implications for allosteric control, making action at a distance an unlikely mechanism by which binding could influence reactivity. However, modular stability may help facilitate the generation of diversity via genetic mechanisms such as duplication, deletion, and recombination, since high modularity would mean that repeats could tolerate substitution of neighboring repeats without deleterious effect on stability.

Despite the modular architecture of ankyrin repeat proteins, the data presented here indicate that the ankyrin repeats of Notch do not possess modular stability. Urea-induced unfolding curves have sigmoidal, cooperative shapes. Moreover, CD and fluorescence describe identical transitions. Such behavior is consistent with long-range cooperativity across the ankyrin domain. Furthermore, for Nank1−7*, the calorimetric enthalpy of unfolding closely matches that obtained using two-state models to fit thermal unfolding monitored

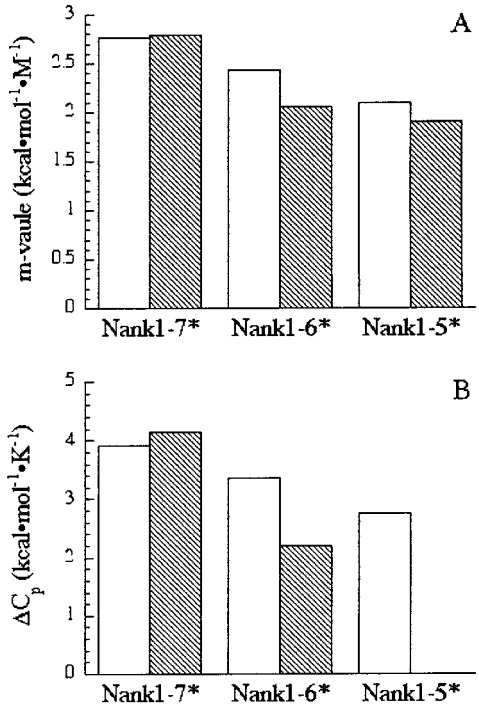

FIGURE 5: Comparison of changes in *m*-value and ΔC_p for unfolding of ankyrin repeat polypeptides of *Drosophila* Notch protein with those predicted from chain length. (A) Measured (stippled bars) and calculated (open bars) *m*-values for urea-induced unfolding. (B) Measured (stippled bars) and calculated (open bars) ΔC_p values for unfolding. Calculated values are based on the linear relations of Myers et al. (*50*) between *m*-value and ΔC_p, and the change in accessible surface area upon unfolding. Changes in accessible surface area upon unfolding are estimated using the linear relation between chain length and ΔASA in Figure 1 of Myers et al. (*50*). Chain lengths of 246, 214, and 181 residues were used for Nank1−7*, Nank1−6*, and Nank1−5*, respectively.

by CD, indicating an all-or-none, cooperative transition in which distant regions of the chain are coupled to each other. To our knowledge, this is the first time the unfolding of a repeat protein has been shown to be two-state by this rigorous thermodynamic criterion.

In addition to affecting the apparent stability and reaction order, modular stability would also affect derivative thermodynamic quantities. If individual repeats unfolded independently, the small cooperative unit size (33 residues) would result in shallow unfolding curves. Two thermodynamic parameters that are sensitive to the amount of surface area exposed during an unfolding transition, and thus, to good approximation, to the size of the cooperative unit, are the *m*-value (*47*, *49*, *50*) and ΔC_p (*50*, *53*, *54*). In a survey of *m*- and ΔC_p values for two-state unfolding of globular proteins, Myers et al. observed a direct linear relation between these thermodynamic quantities and the estimated change in accessible surface area upon unfolding (*50*). The values of *m* and ΔC_p obtained for unfolding of Nank1−7* agree quite well with those predicted for proteins of equivalent length from the relations found by Myers et al. (Figure 5). The predicted *m*-value for urea denaturation is 2.77 kcal•mol^{-1}•M^{-1}, compared with an average *m*-value from CD and fluorescence unfolding of 2.79 kcal•mol^{-1}•M^{-1}. Likewise, the predicted ΔC_p value is 3.92 kJ•mol^{-1}•K^{-1}, compared with a measured value of 4.14 kcal•mol^{-1}•K^{-1}. If the unfolding transitions measured here were those of thermodynamically independent modules, much lower *m*- and

ΔC_p values would be expected, based on the relations of Myers et al. (approaching 0.59 kcal·mol^{-1}·M^{-1} and 0.16 kcal·mol^{-1}·K^{-1}, respectively, for a cooperative unit of 33 residues).

The observation that inclusion of the putative seventh ankyrin repeat shifts the unfolding transition to higher denaturant concentration and temperature (Figures 1, 3, and 4) provides further evidence for long-range cooperative interactions in the Notch ankyrin domain. If the putative seventh repeat adopted a fold of intrinsically high stability, but did not influence the stability of the other repeats, its inclusion in the ankyrin domain would produce a broader unfolding transition, consisting of a large contribution from unfolding of the N-terminal repeats under mildly destabilizing conditions, and a smaller contribution from unfolding of the C-terminal region under strongly destabilizing conditions. Instead, we find that the entire unfolding transition is shifted to strongly destabilizing conditions and is sharpened (Figures 1 and 3). Thus, the entire ankyrin domain (including regions near the N-terminus) is stabilized by the putative seventh, C-terminal repeat.

Differences in Stability of the Notch Ankyrin Polypeptides Containing Variable Numbers of Repeats. The significant increase in stability that results from including the putative seventh ankyrin repeat sequence in the Notch ankyrin domain strongly argues that this region is a critical component of this folding unit. The unfolding free energy of the six-repeat polypeptide is relatively low (4.1 kcal·mol^{-1}) compared with typical values for globular proteins, but is quite similar to unfolding free energies measured for other ankyrin repeat domains. Estimates of the stability of p16, a polypeptide that contains four ankyrin repeats, range from 1.9 to 3.7 kcal·mol^{-1}, with higher salt concentrations yielding higher estimates of stability (*55*−*58*). Although the similar low stabilities of p16 and Nank1−6* might be taken as suggestive that the ankyrin repeat fold is of modest stability overall, perhaps either as a result of a small hydrophobic core or as a result of modularity, the high unfolding free energy and two-state nature of the seven-repeat Notch ankyrin polypeptide (8.0 kcal/mol) demonstrate that these modularly structured proteins can exhibit stabilities similar to those seen for globular proteins of irregular topology.

The small change in stability that results from inclusion of the sixth repeat in the Notch ankyrin domain, compared with the large change resulting from inclusion of the putative seventh repeat, may suggest that different ankyrin repeats contribute different amounts to the overall stability of the domain. Alternatively, these stability differences may result from differences in solvent interactions with the sixth versus seventh repeat. Given the importance of the putative seventh repeat, the sixth repeat is not likely to make contacts with solvent on the surface that is normally packed against the seventh repeat; thus, solvation of this interface may be destabilizing, and may cancel any stabilizing contributions from the sixth repeat. Folded as a seventh ankyrin repeat, the C-terminal region would contain two polar residues at conserved nonpolar positions of helix-1 that would be better suited to contact solvent on its exposed face [Ser at the ϕ7 position, and Arg at the Leu3 position; Figure 1, preceding paper (*43*)]. In this regard, the C-terminal, seventh ankyrin repeat may serve as a "capping" repeat, interacting favorably with the previous repeat on one side and with solvent on the other.

Identification of a seventh ankyrin repeat as part of the Notch ankyrin domain simplifies the interpretation of an interaction detected by a yeast two-hybrid assay between Suppressor of Hairless (a downstream transcription factor in the Notch pathway) and the intracellular domain of the Notch receptor (*37*). This interaction was proposed to involve the region of polypeptide N-terminal to the Notch ankyrin domain (termed RAM), along with the region immediately C-terminal to the six identified Notch ankyrin repeats. The six-repeat ankyrin domain was shown to be necessary but not sufficient for the C-terminal interaction (*37*). Since the results presented here demonstrate that the C-terminal region is a critical part of the ankyrin domain, the Suppressor of Hairless interaction can be regarded as an interaction with two adjacent domains on Notch (the RAM and full ankyrin domains), rather than with two domains separated by a third elongated, ankyrin domain. We expect that the seventh ankyrin repeat will contribute significantly to the surface of the ankyrin domain that binds to Suppressor of Hairless; whether the six N-terminal repeats simply act by stabilizing the seventh repeat, or whether they also contribute directly to the binding interaction must await further studies.

Differences in Derivative Thermodynamic Parameters for Unfolding, and Relation to the Extent of the Structural Transition. In addition to large change in unfolding free energy that results from deletion of the putative seventh ankyrin repeat, there are large changes in quantities that describe sensitivity of stability to the perturbants used to bring about unfolding (urea and temperature). Deletion of the putative seventh repeat results in large changes in the *m*-value, ΔC_p, $\Delta H°$, and $\Delta S°$, whereas further deletion of the sixth repeat produces only a minor change in the *m*-value for urea-induced unfolding. Since the *m*-value and ΔC_p are both believed to be related to surface area changes upon unfolding (*47*, *49*, *50*, *53*, *54*, *59*), these parameters would be expected to increase by roughly one-sixth upon addition of the putative seventh repeat to a domain consisting of six folded repeats. Instead, the *m*-value and ΔC_p increase by roughly 50 and 85%, respectively, when the seventh repeat is added (Figure 5). This large increment suggests that the addition of the putative seventh repeat results in an increase in the number of residues participating in the unfolding reaction beyond residues within the seventh repeat. Likewise, the entropy of unfolding is larger (making unfolding more favorable) for the seven-repeat protein than for the six-repeat protein by around 60%. One source expected to contribute significantly to this difference in entropy of unfolding is an increase in conformational disorder upon unfolding. The 60% increase in unfolding entropy of Nank1−7* compared to Nank1−6* suggests that the putative seventh repeat facilitates an order−disorder transition that extends over a greater portion of the polypeptide chain than just the seventh repeat.

Structural Interpretation of the Thermodynamics of Deleting the Putative Seventh Repeat. All of the structural and thermodynamic data are consistent with a model in which the C-terminal region folds as a seventh ankyrin repeat, and that the sixth repeat is only folded if the seventh repeat is included. As shown in the preceding paper (*43*), deletion of the putative seventh repeat decreases the α-helix content and increases the frictional coefficient of the resulting six-repeat

polypeptide, consistent with unfolding of the sixth repeat. The thermodynamic data here are consistent with this picture. Whereas experimentally determined *m*- and ΔC_p values for unfolding of the seven-repeat protein match expected values surprisingly well, values for the six-repeat protein match values expected for a protein containing only five folded repeats (Figure 5).

In contrast, further deletion of the sixth repeat appears to have little effect on the remaining structure or stability. Fluorescence measurements indicate that the structural features of the environment surrounding the tryptophan in repeat 5 are identical between Nank1−6* and Nank1−5*, indicating a nonpolar, shielding environment surrounding this tryptophan. The axial ratio of the five-repeat polypeptide, as estimated from the frictional coefficient, is significantly decreased compared with the six-repeat construct, consistent with deletion of the disordered repeat without further unfolding of the remaining repeats. Likewise, the sensitivity of the unfolding free energy and *m*-value to removal of the sixth repeat are both small, consistent with an absence of interactions between the sixth repeat with those preceding it, when the seventh repeat is absent. As the sixth repeat matches well to the ankyrin repeat consensus, the origins of the instability of the sixth repeat in the absence of the seventh are not obvious. Although it does not lend structural stability to the ankyrin domain in the absence of the seventh repeat, the sixth repeat may contribute to stability when the seventh repeat is present. Further deletion and mutational studies should provide a better picture of the contributions of each repeat to structure and stability in this biologically important domain.

ACKNOWLEDGMENT

We thank Spyros Artavanis-Tsakonas and Kenji Matsuno for providing us with a Notch cDNA clone. We are grateful to Steven Blacklow for informing us of a potential seventh ankyrin repeat in Notch. We thank David Draper and Cuautemoc Garcia-Garcia for access to and assistance with differential scanning calorimetry, Kip Murphy for helpful discussions about analysis of DSC data and thermal unfolding, and Bertrand Garcia-Moreno E. and Karen Fleming for numerous discussions about the results and interpretations. We thank Debbie Andrew for critically reading this manuscript.

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BI011436+