Supplementary Information

. Materials and Methods

1. Proteins measured

The preparation of recombinant apolipoprotein A-1 has been described before(Zehender et al., 2012). The purified recombinant Apo A-I differed from the human wild-type (wt) Apo A-I by two additional N-terminal glycine residues. Electrospray mass spectrometry revealed a molecular mass of 28192.3 Da (the theoretical value for wt Apo A-I with two Gly residues is 28192.7 Da).The protein concentration was determined by measuring the optical density at 280 nm using an extinction coefficient of ε = 31070 M^{-1} cm^{-1}. Lysozyme (from hen egg white) was obtained from SigmaAldrich (Switzerland).

.2. Circular Dichroism Spectroscopy

CD measurements at different temperatures were made with a Chirascan CD spectrometer (Applied Photophysics Ltd., Leatherhead, U.K.). A quartz cuvette with a path length of 1 mm (or 0.1mm) was used. All spectra were corrected by subtracting the buffer. The percentage of peptide secondary structure was estimated from a computer simulation based on the reference spectra obtained by Reed and Reed(Reed & Reed, 1997).

.3. Differential Scanning Calorimetry (DSC)

DSC scans were made with a VP-DSC (Microcal, Northampton, MA). Protein solutions were degassed and the reference cell contained only buffer. The heating rate was 1 °C/min. DSC scans were made in PBS buffer, pH 7.4. Further evaluation of the DSC curves was performed as described before(Schulthess et al., 2015; Zehender et al., 2012).

Footnotes in all tables

[a] T_0 maximum of the C_p vs. T curve, T_∞ characteristic temperature of the Zimm-Bragg theory.

[b] T_{in} - T_{end} temperature interval of the unfolding transition

[c] $\Delta C^0_{p,NU}$ difference in the molar heat capacity of unfolded vs. native protein

[d] ΔH^0_{NU}, h ΔH^0_{NU} conformational enthalpy (= van't Hoff enthalpy) used in simulating the unfolding transition with the 2-state model; h unfolding parameter of the Zimm-Bragg theory

[e] ΔH^0_{total} black number: total molar enthalpy obtained by integration of the experimental DSC transition between T_{ini} andT_{end} including the contribution of the increased heat capacity $\Delta C^0_{p,NU}$; blue number : best fit of the 2-state model; red number: best fit of the Zimm-Bragg theory

[f] N_{ZB} number of peptide units used in the Zimm-Bragg theory

[g] σ nucleation parameter used in the Zimm-Bragg theory

[h] $T_{0,CD}$ midpoint of unfolding transition measured with CD spectroscopy.

[i] ε_{222nm} mean residue ellipticity at 222 nm and room temperature (native protein)

[k] f_{helix} maximum helix content at room temperature

[l] ΔN_α number of peptide units involved in the helix ("folded") $\rightleftarrows$ coil ("unfolded") transition

Proteins

For the two-state model and the Zimm-Bragg theory it is important that the unfolding reactions are reversible and that the protein is monomeric at all times. The following proteins were all analysed with the two-state model by the corresponding authors and we therefore assume that the criteria of reversibility is fulfilled in all examples.

S1 Aspartate receptor cytoplasmic fragment

Wu, J. R., Long, D. G., and Weis, R. M. (1995) *Biochemistry* **34**, 3056-3065

"Reversible dissociation and unfolding of the Escherichia coli aspartate receptor cytoplasmic fragment"

Figures 2 and 5 were analysed.

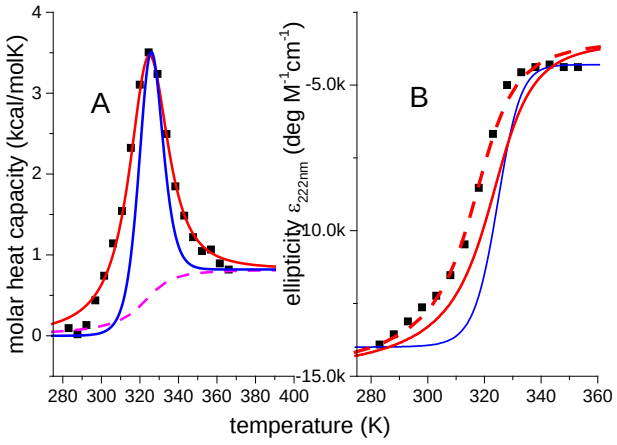

Figure S1. (A) DSC. (□)Experimental data. The protein shows a rather broad transition between 20^0C and 75^0C ($\Delta T = 55^0$C). Solid red line: Zimm-Bragg theory.Simulation with the parameters given in table S1 below. Solid blue line: two-state model. Dashed magenta line is the contribution of the heat capacity term $\Delta C^0_{p,NU}$ to the unfolding reaction, calculated with the Zimm-Bragg theory.

(B) (□) Experimental data obtained with CD spectroscopy. Solid red line: Zimm-Bragg theory. Solid blue line: two-state model. The same parameters as in panel A were used for the simulation. Red dashed line: the Zimm-Bragg simulation is shifted by 6°C towards lower temperatures.

The Zimm-Bragg theory provides an excellent fit to the DSC and CD spectroscopy data using the same set of parameters for both simulations. The two-state model is clearly less satisfactory.

Table S1

Aspartate receptor cytoplasmic fragment (297 aa, 31 kDa)(Wu et al., 1995)

	T_0, T_∞ [a]	T_{ini}-T_{end} [b]	$\Delta C^0_{p,NU}$ [c]	$\Delta H^0_{NU}, h$ [d]	ΔH^0_{total} [e]	N_{ZB} [f]	σ [g]
	K	K	kcal/molK	kcal/mol	kcal/mol		
DSC Experiment	324	293-348	0.82		107.2		
2-state model	324		0.82	50.2	70.1		
Zimm-Bragg theory	327		0.82	1.1	103.1	100	1×10^{-3}
CD Experiment	$T_{0,CD}$ [h]	ε_{222nm} [i]		f_{helix} [k]		ΔN_α [l]	
	K	$10^3 degM^{-1}cm^{-1}$					
	318	-13.9		0.43		100	

S2 Ubiquitin pH 2.0

Privalov, P. L., and Dragan, A. I. (2007) *Biophys Chem* **126**, 16-24

"Microcalorimetry of biological macromolecules"

Figure 1, pH 2.0 was analysed

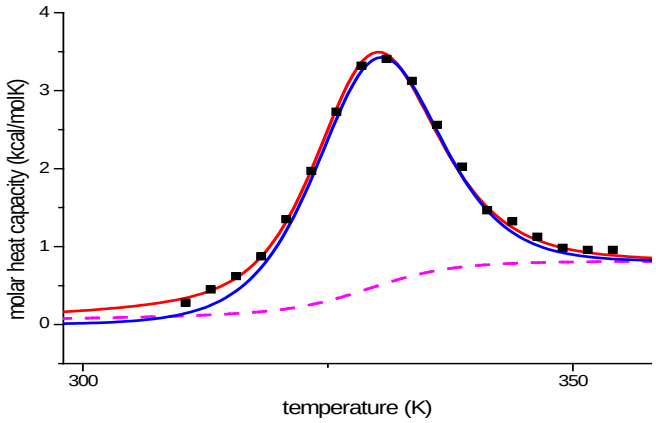

Figure S2. Thermal unfolding of ubiquitin at pH 2.0. (□) Experimental data taken from figure 1 of reference(Privalov & Dragan, 2007). The Zimm-Bragg theory (solid red line) and the 2-state model (solid blue line) provide good descriptions of the DSC transition. The dashed magenta line describes the contribution of $\Delta C^0_{p,NU}$ *to the unfolding process. The simulation parameters are listed in table S2*

The transition temperature of ubiquitin is strongly pH-dependent (fig. 1 of reference. (Privalov & Dragan, 2007)) . The highest transition temperature is observed at the highest pH value. At pH 2.0 the midpoint of the transition is at T_0 = 56°C. The change in heat capacity is $\Delta C^0_{p,NU}$ = 814 cal/molK. The total enthalpy of unfolding between 310K and 351K is ΔH^0_{exp} = 70.8 kcal/mol.

The 2-state model (blue line) provides a good fit of the experimental data with the fit parameters $\Delta H^0_{NU} = \Delta H_{vH}$ 50.1 kcal/mol and $\Delta C^0_{p,NU}$ = 814 cal/molK.

The Zimm-Bragg theory provides an even better fit of the experimental data with σ = $2x10^{-5}$ and N_{ZB} = 70 peptide units participating in the unfolding reaction

In figure 6 of reference (Ibarra-Molero et al., 1999a) one finds additional information on the enthalpy using the 2-state model. At a transition temperature of 56°C the enthalpy is ΔH_{vH} = 47.8 kcal/mol, consistent with the present analysis.

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Table S2

Ubiquitin (76 aa, 8.433 kDa) pH 2.0(Privalov & Dragan, 2007)

	T_0, T_∞ [a]	T_{ini}-T_{end} [b]	$\Delta C^0_{p,NU}$ [c]	$\Delta H^0_{NU}, h$ [d]	ΔH^0_{total} [e]	N_{ZB} [f]	σ [g]
	K	K	kcal/molK	kcal/mol	kcal/mol		
DSC Experiment	329	310-351	0.814		70.8		
2-state model	329		0.814	50.2	68.3		
Zimm-Bragg theory	349		0.814	1.1	70.7	70	2×10^{-5}

S3 Ubiquitin (bovine) pH 3.0

Ibarra-Molero et al. (1999) *Biochemisty* **38**, 8138-49.

"Thermal versus guanidine-induced unfolding of ubiquitin. An analysis in terms of the contributions from charge-charge interactions to protein stability."

Figure 5 was analysed.

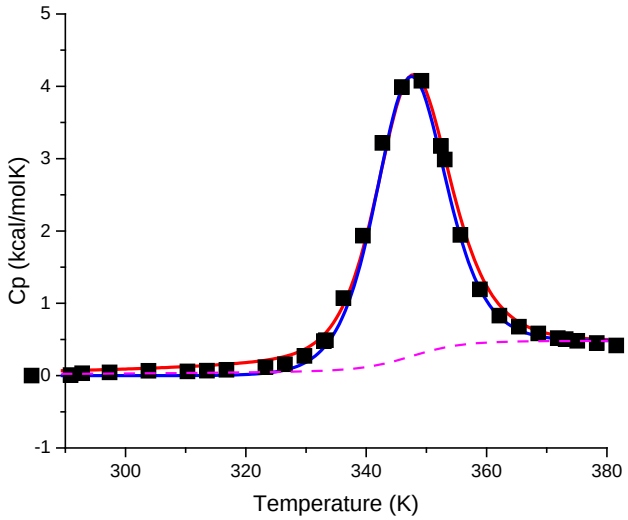

Figure S3. Thermal unfolding of ubiquitin at pH 3.0. (□) Experimental DSC data. The Zimm-Bragg theory (solid red line) and the 2-state model (solid blue line) provide good descriptions of the DSC transition. The dashed magenta line is the contribution of $\Delta C^0_{p,NU}$ *to the unfolding process calculated with the Zimm-Bragg theory .*

At pH 3.0 the midpoint of the transition is at T_0 = 74 °C. The change in heat capacity is $\Delta C^0_{p,NU}$ = 480 cal/molK. The total enthalpy of unfolding between 326 K and 368 K is ΔH^0_{exp} = 76.3 kcal/mol. The 2-state model (blue line) uses $\Delta H^0_{NU} = \Delta H_{vH}$ = 60.9 kcal/mol and $\Delta C^0_{p,NU}$ = 480 cal/molK to simulate the experimental data.

The Zimm-Bragg theory provides a slightly better fit of the experimental data than the 2-state model. The parameters are listed in table 3.

Figure 6 of reference (Ibarra-Molero et al., 1999a) shows a transition temperature of 74^0C at pH 3.0 and a conformational enthalpy of $\Delta H^0_{NU} = \Delta H_{vH}$ =265kJ/mol= 63.4 kcal/mol, consistent with the present evaluation.

Table S3

Ubiquitin (bovine) (76 aa, 8.433 kDa) pH 3.0(Ibarra-Molero et al., 1999a)

	T_0, T_∞ [a]	T_{ini}-T_{end} [b]	$\Delta C^0_{p,NU}$ [c]	$\Delta H^0_{NU}, h$ [d]	ΔH^0_{total} [e]	N_{ZB} [f]	σ [g]
	K	K	kcal/molK	kcal/mol	kcal/mol		
DSC Experiment	347	326-368	0.48		76.3		
2-state model	347		0.48	60.9	71.0		
Zimm-Bragg theory	380		0.48	1.1	76.2	70	2×10^{-6}

S4 Ubiquitin pH 3.0

Privalov, P. L., and Dragan, A. I. (2007) *Biophys Chem* **126**, 16-24.

"Microcalorimetry of biological macromolecules"

Figure 1, pH 3.0 was analysed.

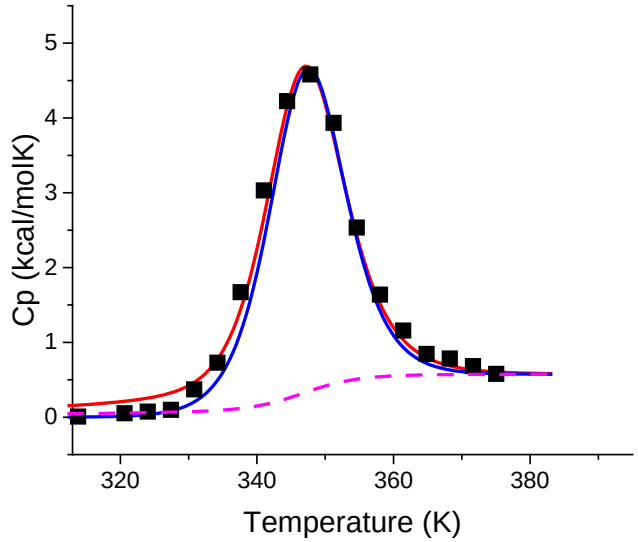

Figure S4. Thermal unfolding of ubiquitin at pH 3.0. (■) Experimental DSC data taken from figure 1 of reference (Privalov & Dragan, 2007). The Zimm-Bragg theory (solid red line) and the 2-state model (solid blue line) provide good simulations of the DSC transition. The dashed magenta line describes the contribution of $\Delta C^0_{p,NU}$ *to the unfolding process. the simulation parameters are listed in table S4.*

At pH 3.0 the midpoint of the transition is found at T_0 = 74°C. The transition curve at pH 3.0 was enlarged and digitised. The change in heat capacity is $\Delta C^0_{p,NU}$ = 573 cal/molK. The total enthalpy of unfolding between 323K and 365K is ΔH^0_{exp} = 84.7 kcal/mol.

Table 1 in (Privalov & Dragan, 2007) lists ΔC_p = 76x33/4.18 = 600 cal/molK and ΔH_t = 76x4.0/4.18 = 72.7 kcal/mol for pH 4.0 and T_0=90 ^{0}C.

Figure 6 of reference (Ibarra-Molero et al., 1999a) shows a transition temperature of 74^0C at pH 3.0 and an enthalpy of $\Delta H^0_{NU} = \Delta H_{vH}$ =265kJ/mol= 63.4 kcal/mol.

Within error limits the data extracted from fig.5 (Ibarra-Molero et al., 1999a) and fig 1 (Privalov & Dragan, 2007) lead to the same results.

Table S4

Ubiquitin (76 aa, 8.433 kDa) pH 3.0(Privalov & Dragan, 2007)

	T_0, T_∞ [a]	T_{ini}-T_{end} [b]	$\Delta C^0_{p,NU}$ [c]	$\Delta H^0_{NU}, h$ [d]	ΔH^0_{total} [e]	N_{ZB} [f]	σ [g]
	K	K	kcal/molK	kcal/mol	kcal/mol		
DSC Experiment	347	323-365	0.573		84.7		
2-state model	347		0.573	64.5	73.7		
Zimm-Bragg theory	374		0.573	1.1	81.2	76	3×10^{-6}

S5 S461L Aspartate receptor cytoplasmic fragment

Wu, J. R., Long, D. G., and Weis, R. M. (1995) *Biochemistry* **34**, 3056-3065.

"Reversible dissociation and unfolding of the Escherichia coli aspartate receptor cytoplasmic fragment."

S461L mutant of aspartate receptor cytoplasmic fragment

DSC scan: figure 3. **CD spectroscopy**: figure 7.

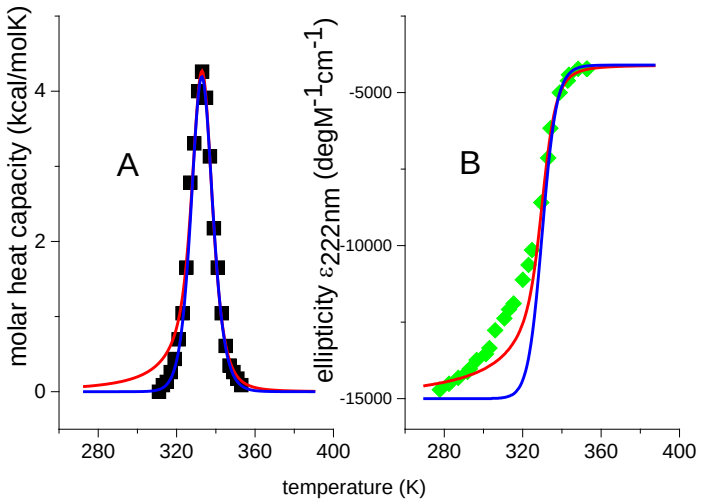

Figure S5. (A) (■)DSC data. The Zimm-Bragg theory (red line) and the 2-state model (blue line) provide good descriptions of the DSC transition. (B) (□) Experimental CD data The CD spectroscopy reveals a biphasic behavior. The theoretical lines were calculated with the same set of parameters as used for the DSC transition but the temperature scale of the simulations was shifted by 2°C towards lower temperatures. Both models fit the high temperature end of the transition equally well but deviate considerably at the low temperature end. The Zimm-Bragg theory (red line) shows a t better fit than the 2-state model (blue line). $\varepsilon_{222nm} = -11000\Theta_N - 4100$. *($\Theta_N$ is the extent of native protein calculated with either the Zimm-Bragg theory or the 2-state model). The simulation parameters are listed in table S5.*

The DSC scan was corrected by the authors such that the increase in the heat capacity was eliminated. The protein was measured in parallel to the wild-type protein. Compared to

the wild-type protein, the DSC transition of S461L is sharper and shifted by about 9°C towards higher temperatures. The midpoint of the transition is at T_0 = 60°C. The total enthalpy of unfolding between 313K and 353K is ΔH^0_{exp} = 64.1 kcal/mol. As the contribution from $\Delta C^0_{p,NU}$ is eliminated by a progressive baseline ΔH^0_{exp} = 64.1 kcal/mol is much lower than expected for the unfolding of N ~ 110 amino acids, the number deduced from the change in the CD spectrum.(For the same reason ΔH^0_{exp} = 64.1 kcal/mol is also much smaller than ΔH^0_{exp} =107.2 kcal/mol found for the wild-type; cf Sup. Inf. 1). The DSC unfolding transition can be simulated equally well with the Zimm-Bragg theory and the 2-state model.

The thermal unfolding of mutant S461L was also measured with CD spectroscopy. Figure 5 in the reference(Wu et al., 1995) and figure 7 in reference(Seeley et al., 1996) show the ellipticity at 222 nm as a function of temperature. Fig. 7 in reference(Seeley et al., 1996) indicates considerable differences in the ellipticity of monomeric versus dimeric. S461L. The theoretical analysis shown below was applied to monomeric S461L (figure 7(Seeley et al., 1996)). The midpoint of the CD-measured unfolding transition is shifted by 3°C towards lower temperatures compared to the maximum of the DSC data. The same parameters as used for DSC provide a good fit of the CD transition.

The unexpected deviation of the CD spectra at low temperatures could be caused by the monomer-dimer equilibrium of this mutant. In contrast, the wild-type protein is always monomeric. The Zimm-Bragg theory yields a better fit to the low-temperature region than the 2-state model.

Table S5

S461L aspartate receptor cytoplasmic fragment (297 aa, 31 kDa)(Wu et al., 1995)

	T_0/T_∞ [a]	T_{ini}-T_{end} [b]	$\Delta C^0_{p,NU}$ [c]	ΔH^0_{NU}, h [d]	ΔH^0_{total} [e]	N_{ZB} [f]	σ [g]
	K	K	kcal/molK	kcal/mol	kcal/mol		
DSC Experiment	333	313-353	0		64.1		
2-state model	333		0	61.6	61.6		
Zimm-Bragg theory	339.5		0	1.1	66.2	110	1×10^{-4}
CD Experiment	$T_{0,CD}$ [h]	ε_{222nm} [i]		f_{helix} [k]		ΔN_α [l]	
	K	$10^3 degM^{-1}cm^{-1}$					
	33	-14.7 – -4.64		0.45		~110	1×10^{-4}

S6 β-lactoglobulin at pH 1.1 -1.5

Garcia-Hernandez et al (1998) *Biochemistry and Molecular Biology International* **45**,761-768 "Spectroscopic and thermodynamic evidence for a complex denaturation mechanism of bovine β-lactoglobulin A."

Figures 1 and 2a, pH 1.1, were evaluated.

β-Lactoglobulin, the major bovine whey protein, is a well characterised globular protein. The monomeric forms of the A and B variants are composed by 162 amino acid residues (MW: 18.4.kDA(Relkin, 1998);18.24 kDa(Garcia-Hernandez et al., 1998)). "The monomer has a core structural pattern consisting of a β-barrel formed by eight strands of anti-parallel β-sheets and another β-strand and α-helix on the surface "(Relkin, 1998).

The secondary structure at 20-25°C and pH 6.7 is composed of 10% α-helix, 50% β-sheet, 8% reversed turn and 35% random coil. At 80°C the corresponding numbers are 2 % α-helix, 42 % β-sheet, 6 % reversed turn and 50 % random coil. At pH 6.7 these structural changes cannot be attributed to a complete thermal unfolding as denaturation requires a temperature above 100°C (see equation 3 in (Jia et al., 1993)). Instead, the structural changes are assigned to the formation of the molten globule structure.

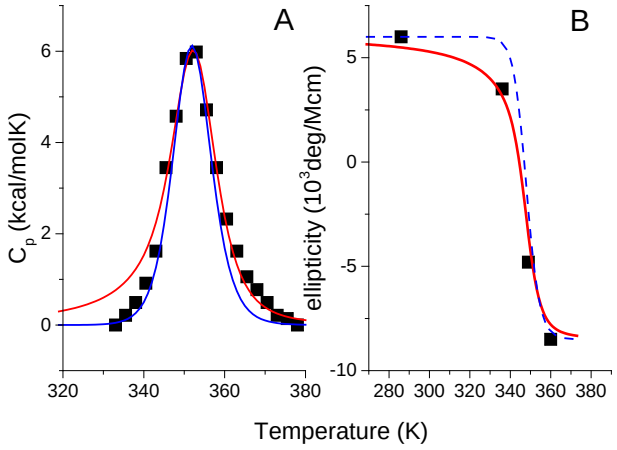

Figure S6. (A) (□)Thermal unfolding of β-lactoglobulin at pH 1.1 measured with DSC. Data taken from fig. 2a (Garcia-Hernandez et al., 1998)and simulated with the Zimm-Bragg theory (red solid line) and the 2-state model (blue dashed line). (B) CD spectroscopy.(□) Mean residue ellipticity at 198 nm and pH 1.5 as a function of temperature (fig. 1a (Garcia-Hernandez et al., 1998)).The CD transition has its midpoint 8°C lower than the DSC transition. Simulation with the Zimm-Bragg theory (red solid line) and the 2-state model (blue dashed line). The same parameters as used in the DSC experiment were applied but the midpoint temperature was shifted by 8°C towards lower temperatures. The 2-state model predicts a sharper CD transition than the Zimm-Bragg theory. The parameters used for the simulations are summarised in table S6.

Simultaneous DSC and CD spectroscopy experiments on β-lactoglobulin are reported in (Garcia-Hernandez et al., 1998). The spectroscopic measurements were performed at pH 1.5 in 10 mM glycine buffer. At this pH, the CD spectra show a well-defined structural change at 76-78°C (cf.fig.1a(Garcia-Hernandez et al., 1998)). Only two different populations were detected by a factor analysis. "The thermal denaturation of β-lactoglobulin can be depicted fairly well as a 2-state process"(Garcia-Hernandez et al., 1998).

In contrast, UV-spectroscopy of the same samples showed a more complex response characterised by 2 isodichroic points. It is suggested that the UV-spectroscopic data detect a stable intermediate emerging from structural modifications restricted to local regions of the native β-lactoglobulin (molten globular?).

Chemical denaturation with 8M urea produces an even more dramatic change of the β-lactoglobulin CD spectrum. This observation argues against a complete unfolding of β-lactoglobulin by thermal denaturation. It is suggested that the thermally denatured β-lactoglobulin retains a significant amount of buried hydrophobic surface area.

Table S6

β-lactoglobulin pH 1.1 (162 aa, 18.24 kDa)

	T_0, T_∞ [a]	T_{ini}-T_{end} [b]	$\Delta C^0_{p,NU}$ [c]	$\Delta H^0_{NU}, h$ [d]	ΔH^0_{total} [e]	N_{ZB} [f]	σ [g]
	K	K	kcal/molK	kcal/mol	kcal/mol		
DSC Experiment	352.5	333-373	0		92.5		
2-state model	352.5		0	76.4	76.4		
Zimm-Bragg theory	356.3		0	1.1	98.8	142	1.5×10^{-4}
CD Experiment	$T_{0,CD}$ [h]	ε_{222nm} [i]		f_{helix} [k]		ΔN_α [l]	
	K	$10^3 degM^{-1}cm^{-1}$					
	344.5	6 – -5.4		0.08			

S7 β-lactoglobulin at pH 2.5

Garcia-Hernandez et al (1998) *Biochemistry and Molecular Biology International* **45**,761-768 "Spectroscopic and thermodynamic evidence for a complex denaturation mechanism of bovine β-lactoglobulin A."

Figure 2a, pH 2.5, was evaluated.

The molecular weight of β-lactoglobulin A was taken as 18.24 kDa(Garcia-Hernandez et al., 1998).Thermal unfolding transitions were measured with DSC for different pH values between 1.1 and 2.5 (fig. 2a(Garcia-Hernandez et al., 1998)) . Unfortunately, a progressive baseline was subtracted that cancelled the increase in heat capacity. These 'truncated' thermodynamic data are summarised in table 1 of reference (Garcia-Hernandez et al., 1998). Excluding the data points at pH 1.0, the transition temperature T_m as well as the transition enthalpy ΔH_{cal} are found to depend linearly on the pH .

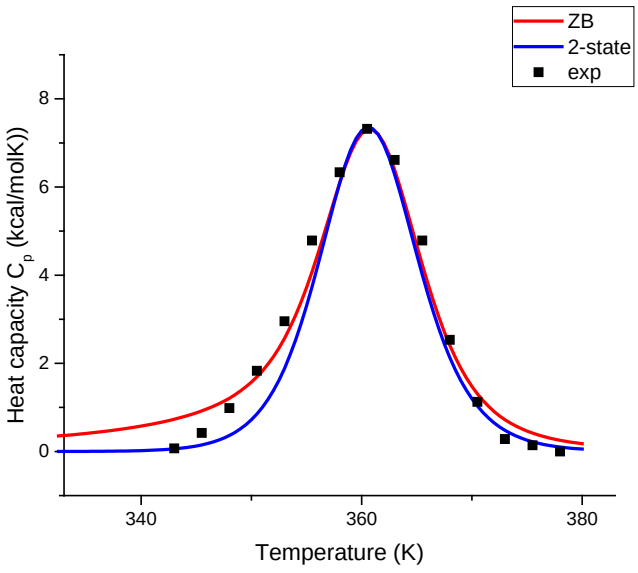

Figure S7. Thermal unfolding of β-lactoglobulin at pH 2.5 measured with DSC (□). Data taken from fig. 2a (Garcia-Hernandez et al., 1998) and simulated with the Zimm-Bragg

theory (red solid line) and the 2-state model (blue dashed line). The parameters used for the simulations are listed in table S7.

The parameters used for the simulation are summarised in the following table.

Table S7

β-lactoglobulin pH 2.5 (162 aa, 18.24 kDa)

	T_0/T_∞ [a]	T_{ini}-T_{end} [b]	$\Delta C^0_{p,NU}$ [c]	ΔH^0_{NU}, h [d]	ΔH^0_{total} [e]	N_{ZB} [f]	σ [g]
	K	K	kcal/molK	kcal/mol	kcal/mol		
DSC Experiment	360.7	343-378	0		96.0		
2-state model	360.7		0	87.2	87.2		
Zimm-Bragg theory	367		0	1.1	97.4	142	5×10^{-5}

S8 β-lactoglobulin at pH 3.3

Schwarz, F.P. (1990) *Thermochimica Acta* 159, 305-325.

"Biological thermodynamic data for the calibration of the differential scanning calorimeters: heat capacity data on the unfolding transition of β-lactoglobulin in solution".

Figure 1b was analysed.

For introductory information on β-Lactoglobulin see S6.

The analysis is based on figure 1B of reference (Schwarz, 1990) The DSC experiment was performed in 0.2 M glycine buffer at pH 3.3. The midpoint of the transition is listed in table 1 of this reference as T_m = 360 K= 87°C. Table 1 also gives ΔH_{vH} = 444 kJ/mol = 106.2 kcal/mol and $\Delta C^0_{p,NU}$ = 19.3 kJ/ molK = 4.59 kcal/mol K. The increase in heat capacity is unusually large and the author concludes from a large number of measurements with β-lactoglobulin: "The average value of $\Delta C^0_{p,NU}$ is 12.7±9.2 kJ/molK which is within the range of 4-7 kJ/molK obtained by Privalov and Khechinashvili(Privalov & Khechinashvili, 1974) for globular proteins in HCl-lysine buffer solutions"(Schwarz, 1990). No CD spectra were measured.

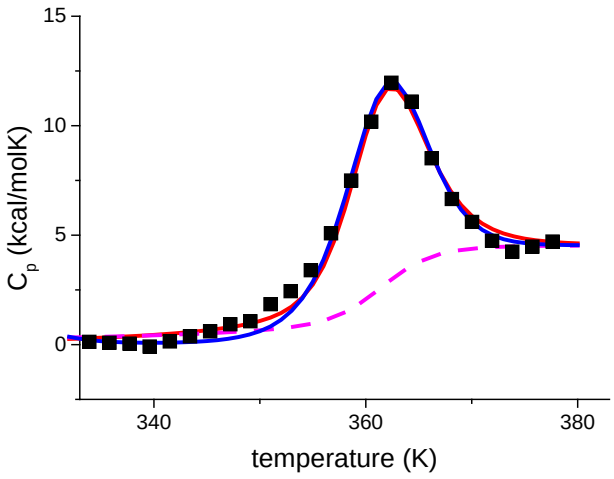

Figure S8. Thermal unfolding of β-lactoglobulin at pH 3.3 measured with DSC. (□). Data taken from fig. 1B(Schwarz, 1990). Zimm-Bragg theory (red solid line) and the 2-state model

(blue solid line). The dashed magenta line shows the contribution of the heat capacity term $\Delta C^0_{p,NU}$ *to the transition enthalpy, calculated with the Zimm-Bragg theory. The parameters used in the simulations are listed in table S8.*

The evaluation of figure 1B (Schwarz, 1990) yields $T_m = 87.6°C$ for the maximum of the Cp vs. T transition curve and $\Delta C^0_{p,NU}$ =4.545 kcal/mol = 19 kJ/molK. The total calorimetric heat evaluated from figure 1B is ΔH^0_{exp} = 655 kJ/mol = 156.5 kcal/mol for the temperature interval 333-373 K. The contribution of the $\Delta C^0_{p,NU}$ term to the unfolding enthalpy is indicated by the dashed magenta line (calculated with the Zimm-Bragg theory) and amounts to $\Delta H^0_{C_p,NU}$ = 67.9 kcal/mol = 284 kJ/mol, resulting in $\Delta H^0_{NU,ZB}$ = 87.9 kcal/mol = 368 kJ/mol for the conformational enthalpy. The simulation of the unfolding transition with the 2-state model yields a conformational enthalpy of $\Delta H^0_{NU,2-state}$ = 94.3 kcal/mol = 395 kJ/mol.

For the analysis with the Zimm-Bragg theory we make the assumption that the transition from a folded to an unfolded peptide unit is characterised by the average enthalpy of 1.1 kcal/mol. Based on a total unfolding enthalpy of 156.5 kcal/mol it follows that 142 peptide units participate in the unfolding reaction. An excellent fit of the calorimetric transition curve was indeed obtained with n= 142 and a cooperativity parameter of $\sigma = 5\times10^{-6}$. The Zimm-Bragg theory clearly shows that are the unfolding transition of β-lactoglobulin is a highly cooperative processes. The Zimm-Bragg theory and the 2-state model provide almost equally good fits of the experimental unfolding transition.

Table S8

β-lactoglobulin (162 aa; 18.24 kDa)

	T_0, T_∞ [a]	T_{ini}-T_{end} [b]	$\Delta C^0_{p,NU}$ [c]	$\Delta H^0_{NU}, h$ [d]	ΔH^0_{total} [e]	N_{ZB} [f]	σ [g]
	K	K	kcal/molK	kcal/mol	kcal/mol		
DSC Experiment	360.7	333-373	4.538		156.5		
2-state model	360.7		4.538	94.3	148.3		
Zimm-Bragg theory	372.7		4.538	1.1	155.7	142	5×10^{-6}

S9 S44[A] T4 lysozyme pH 4.0

Carra, J. H., Murphy, E. C., and Privalov, P. L. (1996) *Biophys J* **71**, 1994-2001

"Thermodynamic effects of mutations on the denaturation of T4 lysozyme"

Figures 3 and 5, pH 4.0 were analysed.

The S44[A] mutant of T4 lysozyme exhibits a broad transition of $\Delta T \approx 35°C$ compared to $\Delta T \approx 17°C$ for the wild-type protein.

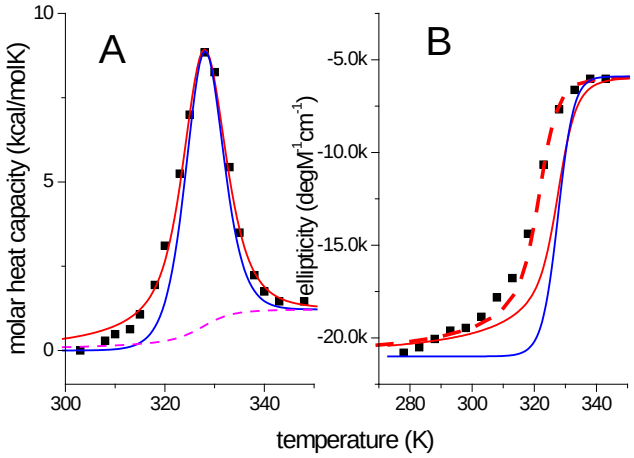

Figure S9. (A) DSC transition. The Zimm-Bragg theory (solid red line) provides a perfect fit of the DSC scan(■). The 2-state model (solid blue line) deviates distinctly from the experimental data. The dashed magenta line shows the contribution of the heat capacity term $\Delta C^0_{p,NU}$ to the transition, calculated with the Zimm-Bragg theory . (B)(□) Experimental data. The discrepancy between the two simulations is even larger for the CD transition if the parameters of the DSC experiment are applied. The 2-state model (blue line) predicts a much sharper transition than is experimentally found. The Zimm-Bragg theory (solid red line) predicts a distinctly broader transition. If shifted by 6°C towards lower temperatures (dashed red line) it provides an almost perfect fit to the experimental data. The parameters used in the simulations are listed in table S9.

The heat capacity function of the S44[A] protein at pH 4.0 is shown in figure 3 of reference (Carra et al., 1996). The unfolding transition is broader than that of wild-type T4

lysozyme and the midpoint is at T_0 = 327.6 K. The total area under the experimental heat capacity curve between $308K \leq T \leq 343K$ is ΔH^0_{exp} = 551.9 kJ/mol = 131.8 kcal/mol. The simulation of the DSC scan with the Zimm-Bragg theory yields $\Delta H^0_{calc,ZB}$ = 527.1 kJ/mol = 126.1 kcal/mol, in agreement with the experimental result. $\Delta H^0_{calc,ZB}$ comprises a contribution of 85.6kJ/mol = 20.4 kcal/mol for the change in the molar heat capacity ($\Delta C^0_{p,NU}$ = 5.1 kJ mol^{-1} K^1 = 1.218 kcal $mol^{-1}K^{-1}$). The conformational change proper is thus characterized by an unfolding enthalpy of ΔH^0_{NU} = 441.4 kJ/mol = 105.6 kcal/mol.

A fit of the experimental data with the 2-state model requires a superposition of two 2-state models according to (Carra et al., 1996). figure S9 shows the simulation with the single two-state model. The conformational enthalpy is ΔH^0_{NU} = 83.6 kcal/mol.

CD spectroscopy (figure 5(Carra et al., 1996)) reveals a broad transition and the shape of the transition curve is well-reproduced by the Zimm-Bragg theory using the same set of parameters as used in the simulation of the DSC experiments (solid red line). The midpoint of the spectroscopic transition is shifted by 6°C towards lower temperatures compared to the calorimetric transition. If the Zimm-Bragg calculation is shifted by 6°C (dashed red line) an almost perfect agreement between experiment and prediction is obtained. In contrast, the 2-state model predicts a much sharper transition and provides only a poor fit of the CD data.

A good fit of the CD-measured unfolding transition is obtained with the 2-state model if the conformational enthalpy is reduced to 38 kcal/mol.

Table S9

S44[A] T4 lysozyme (165 aa, 19.0 kDa) pH 4.0(Carra et al., 1996)

	T_0/T_∞ [a]	T_{ini}-T_{end} [b]	$\Delta C^0_{p,NU}$ [c]	ΔH^0_{NU}, h [d]	ΔH^0_{total} [e]	N_{ZB} [f]	σ [g]
	K	K	kcal/molK	kcal/mol	kcal/mol		
DSC Experiment	327.6	308-343	1.218		131.8		
2-state model	327.6		1.218	83.6	102.1		
Zimm-Bragg theory	334.5		1.218	1.1	126.1	120	5×10^{-5}
CD Experiment	$T_{0,CD}$ [h]	ε_{222nm} [i]		f_{helix} [k]		ΔN_α [l]	
	K	$10^3 degM^{-1}cm^{-1}$					
	321.6	-20.08 – -6.03		0.61		120	

S10 RNAse

Rösgen, J., and Hinz, H. J. (2000), *Biophys Chem 83*, 61-71

"Response functions of proteins"

Figure 1a, pH 2.5 was analysed

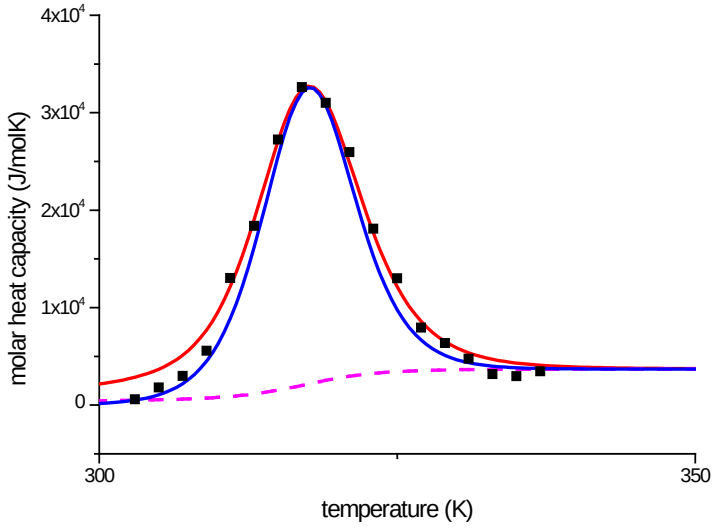

Figure S10. (□) Experimental DSC data. The Zimm-Bragg theory (red line) provides a better description of the DSC curve than the 2-state model (blue line). The dashed line describes the contribution of ΔC_p to the unfolding process calculated with the Zimm-Bragg theory. Simulation parameters listed in table S10.

Table S10

RNAse (124 aa, 13.7 kDa)(Rosgen & Hinz, 2000)

	T_0, T_∞ [a]	T_{ini}-T_{end} [b]	$\Delta C^0_{p,NU}$ [c]	$\Delta H^0_{NU}, h$ [d]	ΔH^0_{total} [e]	N_{ZB} [f]	σ [g]
	K	K	kcal/molK	kcal/mol	kcal/mol		
DSC Experiment	317.3	303-333	0.884		101.5		
2-state model	317.3		0.884	76.4	90.3		
Zimm-Bragg theory	332		0.884	1.1	103.9	90	1×10^{-5}

Table 1 of reference (Rosgen & Hinz, 2000) lists the following data:

T0 = 317.8K, ΔH^0_{NU} = 318 kJ/mol = 76.3 kcal/mol, ΔC_p =3.7kJ/molK=0.885 kcal/molK

S11 Lysozyme pH 1.9

Rösgen, J., and Hinz, H. J. (2000), *Biophys Chem* **83**, 61-71

"Response functions of proteins"

Figure 2a, pH 2.5 was analysed

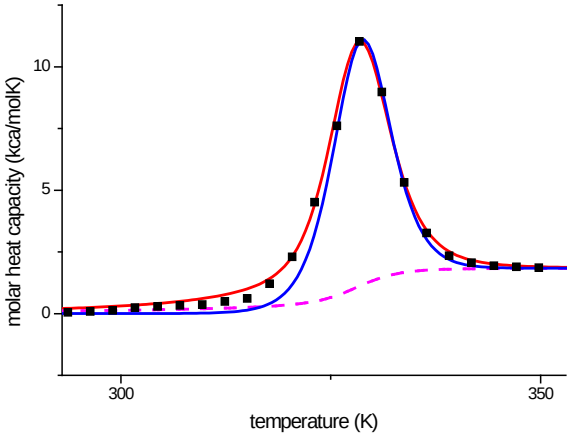

Figure S11. (□) Experimental DSC data. The Zimm-Bragg theory (red line) provides a better description of the DSC curve than the 2-state model (blue line). The dashed magenta line describes the contribution of ΔC_p to the unfolding process, calculated with the Zimm-Bragg theory. Simulation parameters listed in table S11.

Table S11

Lysozyme (129 aa, 14.3 kDa) pH 1.9(Rosgen & Hinz, 2000)

	T_0/T_∞ [a]	T_{ini}-T_{end} [b]	$\Delta C^0_{p,NU}$ [c]	ΔH^0_{NU}, h [d]	ΔH^0_{total} [e]	N_{ZB} [f]	σ [g]
	K	K	kcal/molK	kcal/mol	kcal/mol		
DSC Experiment	328.3	313-341.5	1.839		132.7		
2-state model	328.3		1.839	92.7	117.2		
Zimm-Bragg theory	336		1.839	1.1	132.6	129	2×10^{-5}

Table 1 of (Rosgen & Hinz, 2000) yields the following data: T_0 = 328.3K, ΔH^0_{NU} = 374 kJ/mol = 89.5 kcal/mol, ΔC_p = 6.1kJ/molK =1.46 kcal/molK

S12 Ubiquitin (bovine) pH 4.0

Ibarra-Molero et al. (1999), *Biochim.Biophys.Acta* **1429**,*384-390*

"Cold denaturation of ubiquitin"

Figure 1 was analysed.

At pH 4.0 the midpoint of the transition is at T_0 = 90 °C. The change in heat capacity is ΔC_p = 740 cal/molK. The total enthalpy of unfolding between 350 K and 378 K is ΔH^0_{exp} = 83.8 kcal/mol. The van't Hoff enthalpy used to calculate the DSC unfolding transition with the 2-state model is ΔH^0_{NU} = 70.9 kcal/mol.

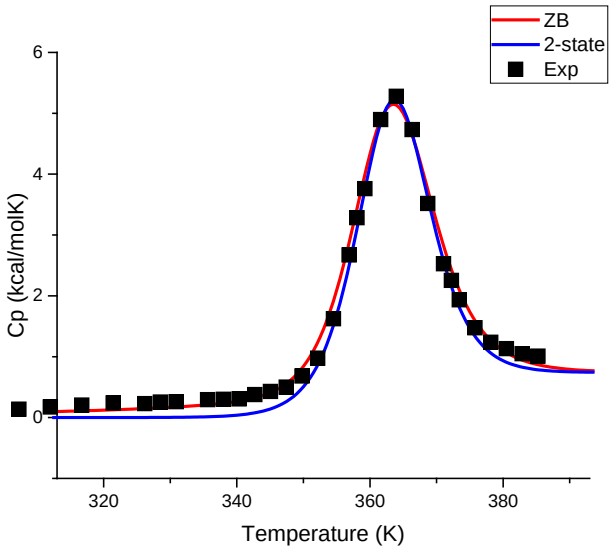

Figure S12. Thermal unfolding of ubiquitin at pH 4.0. (□) Experimental DSC data taken from figure 1 of reference (Ibarra-Molero et al., 1999b). The Zimm-Bragg theory (red line) and the 2-state model (blue line) provide good descriptions of the DSC transition. The dashed magenta line describes the contribution of ΔC_p to the unfolding process. Simulation parameters listed in table S12.

In figure 6 of reference (Ibarra-Molero et al., 1999a) one finds additional information on the enthalpy using the 2-state model. At the transition temperature of 90°C (pH 4.0), the

enthalpy is $\Delta H_{vH} = \Delta H^0_{NU}$ =298kJ/mol= 71.4 kcal/mol, consistent with the present interpretation of figure 1B.

TableS12

Ubiquitin (bovine) (76 aa, 8.433 kDa) pH 3.0(Ibarra-Molero et al., 1999b)

	T_0, T_∞ [a]	T_{ini}-T_{end} [b]	$\Delta C^0_{p,NU}$ [c]	$\Delta H^0_{NU}, h$ [d]	ΔH^0_{total} [e]	N_{ZB} [f]	σ [g]
	K	K	kcal/molK	kcal/mol	kcal/mol		
DSC Experiment	363	350-378	0.74		83.8		
2-state model	363		0.74	70.9	82.0		
Zimm-Bragg theory	398		0.74	1.1	84.8	76	1×10^{-6}

S13 Ubiquitin pH 4.0

Privalov, P. L., and Dragan, A. I. (2007), *Biophys Chem* **126**, 16-24.

"Microcalorimetry of biological macromolecules"

Figure 1, pH 2.0 was analysed.

Figure1 of reference (Privalov & Dragan, 2007)shows that the transition temperature of ubiquitin is strongly pH-dependent. The highest transition temperature is observed at the highest pH value. At pH 4.0 the midpoint of the transition is at T_0 = 90°C. The DSC transition curve at pH 4.0 was enlarged and digitised. The change in heat capacity is $\Delta C^0_{p,NU}$ = 597 cal/molK. The total enthalpy of unfolding between 345K ≤ T ≤ 378K is ΔH^0_{exp} = 90.2 kcal/mol.

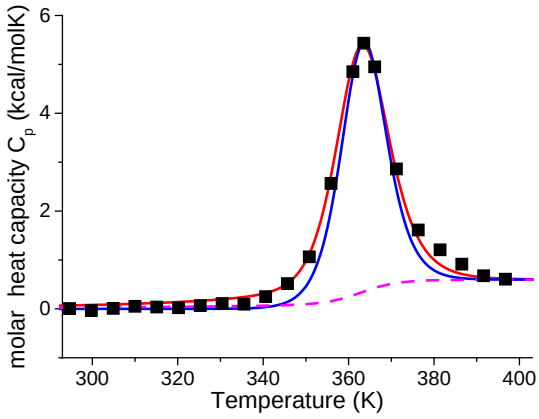

Figure S13. Thermal unfolding of ubiquitin at pH 4.0. (□) Experimental DSC data taken from figure 1 of reference(Privalov & Dragan, 2007). The Zimm-Bragg theory (red line) and the 2-state model (blue line) provide good descriptions of the DSC transition. The dashed magenta line describes the contribution of $\Delta C^0_{p,NU}$ to the unfolding process. Simulation parameters listed in table S13.

The 2-state model (blue line) used $\Delta H^0_{NU} = \Delta H_{vH}$ = 73.3 kcal/mol and $\Delta C^0_{p,NU}$ = 597 cal/molK to simulate the experimental data. In figure 6 of reference (Ibarra-Molero et al., 1999a) one finds additional information on the enthalpy using the 2-state model. At the transition temperature of 90°C, that is, the transition temperature at pH 4.0, the enthalpy as deduced from figure 6 of this reference is ΔH_{vH} = 71.8 kcal/mol, consistent with the present analysis.

Table S13

Ubiquitin (76 aa, 8.433 kDa) pH 4.0

	T_0/T_∞ [a]	T_{ini}-T_{end} [b]	$\Delta C^0_{p,NU}$ [c]	ΔH^0_{NU}, h [d]	ΔH^0_{total} [e]	N_{ZB} [f]	σ [g]
	K	K	kcal/molK	kcal/mol	kcal/mol		
DSC Experiment	363	345-378	0.597		90.2		
2-state model	363		0.597	73.3	89.3		
Zimm-Bragg theory	395		0.597	1.1	92.5	76	2×10^{-6}

S14 Lysozyme (hen egg white) pH 2.5

Privalov, G., Kavina, V., Freire, E., and Privalov, P. L. (1995), *Anal Biochem 232*, 79-85.

"Precise scanning calorimeter for studying thermal properties of biological macromolecules in dilute solution"

Figure 7 was analysed.

Figure 6 and figure 7 of reference (Privalov et al., 1995) show two different DSC unfolding curves of lysozyme . The unfolding of lysozyme depends on the pH of the solution. Increasing the pH shifts the maximum of the C_p vs. T curve towards higher temperatures. (See figure 5 in reference (Privalov, 1997)). The experiment described in figure 6 was made at pH 2.5 in 20 mM glycine buffer (legend to figure 3 (Privalov et al., 1995)). No pH is specified in the legend to figure 7. The midpoint temperature in both figures is T_0=63.5°C. However, the numerical evaluation of the areas under the DSC curves yields ΔH^0_{exp} = 149.5 kcal/mol for figure 7 compared to 136 kcal/mol for figure 6. The DSC experiments were not accompanied by CD measurements. We therefore include our own CD experiments performed under similar conditions (pH 2.5, 20 mM glycine buffer).

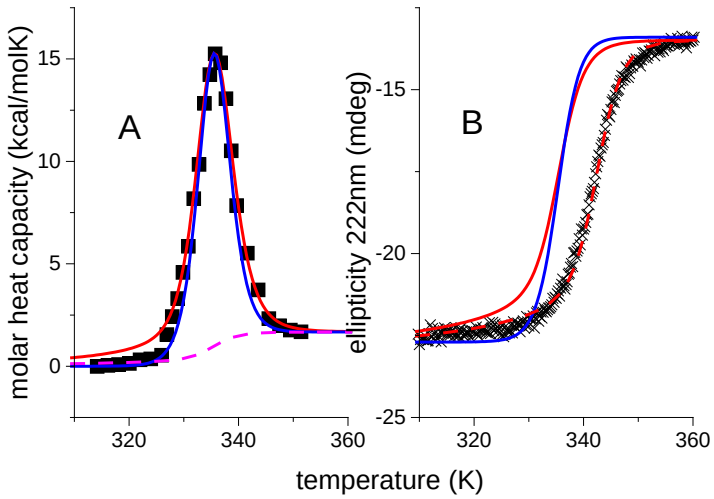

Figure S14. (A) (□)DSCdata of fig.7(Privalov et al., 1995). The Zimm-Bragg theory (red line) and the 2-state model (blue line) provide good fits of the DSC transition. The dashed line describes the contribution of $\Delta C^0_{p,NU}$ *to the unfolding process. (B)(x) CD spectroscopy.*

Ellipticity (mdeg) measured at 222 nm. The solid lines were calculated with the same set of parameters as used for the DSC simulations . The dashed red line corresponds to the solid red line but shifted by 7°C towards higher temperatures. Simulation parameters listed in table S14.

Table S14

Lysozyme (129 aa, 14.3 kDa) pH 2.5 (Fig. 7)(Privalov et al., 1995)

	T_0, T_∞ [a]	T_{ini}-T_{end} [b]	$\Delta C^0_{p,NU}$ [c]	$\Delta H^0_{NU}, h$ [d]	ΔH^0_{total} [e]	N_{ZB} [f]	σ [g]
	K	K	kcal/molK	kcal/mol	kcal/mol		
DSC Experiment	336.8	323-347.5	1.672		149.5		
2-state model	336.8		1.672	113.2	131.2		
Zimm-Bragg theory	350		1.672	1.1	147.9	129	2×10^{-6}

S15 Lysozyme (hen egg white) pH 2.5

Privalov, G., Kavina, V., Freire, E., and Privalov, P. L. (1995) *Anal Biochem 232*, 79-85.

"Precise scanning calorimeter for studying thermal properties of biological macromolecules in dilute solution"

Figure 6 was analysed.

Figure 6 and figure 7 of reference (Privalov et al., 1995) show two different DSC unfolding curves of lysozyme . The unfolding of lysozyme depends on the pH of the solution. Increasing the pH shifts the maximum of the C_p vs. T curve towards higher temperatures. (See figure 5 in reference (Privalov, 1997)). The experiment described in figure 6 was made at pH 2.5 in 20 mM glycine buffer (legend to figure 3 (Privalov et al., 1995)). The midpoint temperature is T_0=63.5°C. The numerical evaluation of the area under the DSC curve yields ΔH^0_{exp} = 136 kcal/mol. The DSC experiments were not accompanied by CD measurements. We therefore include our own CD experiments performed under similar conditions (pH 2.5, 20 mM glycine buffer).

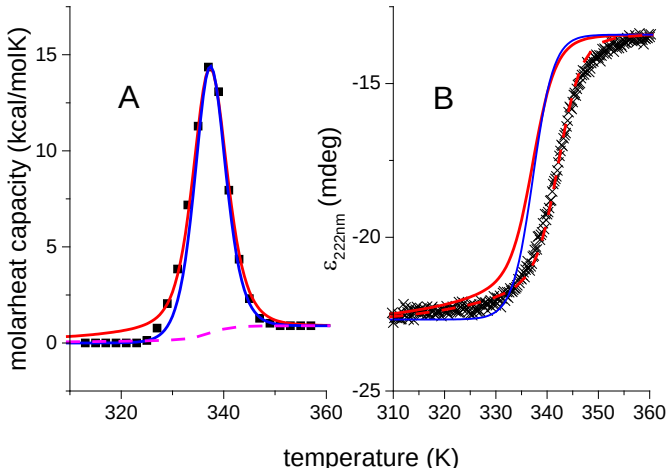

Figure S15. (A) (□) Experimental DSc data derived from figure 6(Privalov et al., 1995). The Zimm-Bragg theory (red line) and the 2-state model (blue line) provide good simulations of

the DSC transition. The dashed line describes the contribution of $\Delta C^0_{p,NU}$ to the unfolding process. (B) CD spectroscopy. Ellipticity (mdeg) measured at 222 nm (x). The solid lines were calculated with the same set of parameters as used for the simulation of the DSC transition curves. The dashed line corresponds to the solid red line but shifted by 6°C towards higher temperatures. Simulation parameters listed in table S10.

Lysozyme (129 aa, 14.3 kDa) pH 2.5 (Fig.6)

	T_0, T_∞ [a]	T_{ini}-T_{end} [b]	$\Delta C^0_{p,NU}$ [c]	$\Delta H^0_{NU}, h$ [d]	ΔH^0_{total} [e]	N_{ZB} [f]	σ [g]
	K	K	kcal/molK	kcal/mol	kcal/mol		
DSC Experiment	336.5	323-347	2.317		136		
2-state model	336.8		2.317	104.6	129.2		
Zimm-Bragg theory	351		2.317	1.1	134.9	129	1×10^{-6}

S16 Myoglobin (sperm whale metmyoglobin)

Privalov, P.L. et al.(1986) *J. Mol. Biol.**190***, 487-498

“Cold denaturation of myoglobin”

Figure 3b , pH 10.7 was analysed.

Myoglobin is a small globular protein of molecular weight 17,900 Da. The crystal structure shows eight different right-handed α-helices that involve 121 out of a total of 153 amino acid residues. The folding/unfolding transition of myoglobin is highly cooperative. A thermodynamic formalism has been presented describing quantitatively the folding behavior of myoglobinin at a hierarchical order of folding units(Freire & Murphy, 1991).

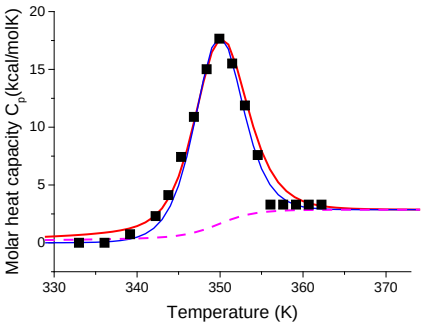

Figure S16. The experimental DSC data (□) of myoglobin are described almost equally well by the Zimm-Bragg theory (red solid line) and the 2-state model (blue solid line). The dashed magenta line shows the contribution of the heat capacity term $\Delta C^0_{p,NU}$ to the transition calculated with the Zimm-Bragg model Simulation parameters listed in table S16.

The folding/unfolding thermodynamics of myoglobin as a function of pH and buffer conditions as being investigated extensively with DSC Under acidic conditions (pH < 5.0) the transition temperature and transition enthalpy *increase* with increasing pH. Under basic conditions (pH > 10.0) the transition temperature and transition enthalpy *decrease* with increasing pH. (See table 1 in (Privalov et al., 1986)). The analysis is based on figure 3B, pH 10.7. The numerical integration of the area under the DSC scan yields ΔH^0_{exp} = 153.8 kcal/mol.

Inspection of table 1 predicts (interpolated) a transition temperature of T_0 = 76.5°C and an unfolding enthalpy ΔH^0_{NU} =.580 kJ/mol = 138.8 kcal/mol. The analysis of the Zimm-Bragg theory yields T_0 = 74.4°C , $\Delta H^0_{calc,ZB}$ = 157.5 kcal/mol , $\Delta H^0_{C_p,NU}$ = 23.82 kcal/mol and ΔH^0_{NU} =133.7 kcal/mol.

Table S16

Myoglobin (153 aa, 17.9 kDa

	T_0, T_∞ [a]	T_{ini}-T_{end} [b]	$\Delta C^0_{p,NU}$ [c]	ΔH^0_{NU}, h [d]	ΔH^0_{total} [e]	N_{ZB} [f]	σ [g]
	K	K	kcal/molK	kcal/mol	kcal/mol		
DSC Experiment	349.5	333-356	2.866		153.8		
2-state model	349.5		2.866	125.4	144.5		
Zimm-Bragg theory	364		2.866	1.1	157.5	140	1×10^{-6}

S17 Pseudo WT T4 lysozyme pH 3.0

Carra, J. H., Murphy, E. C., and Privalov, P. L. (1996) *Biophys J 71*, 1994-2001

"Thermodynamic effects of mutations on the denaturation of T4 lysozyme"

The calorimetric heat was measured in a differential scanning calorimeter at pH 3.0 for pseudo wild-type T4 lysozyme. In parallel, the CD spectra were measured under the same conditions.

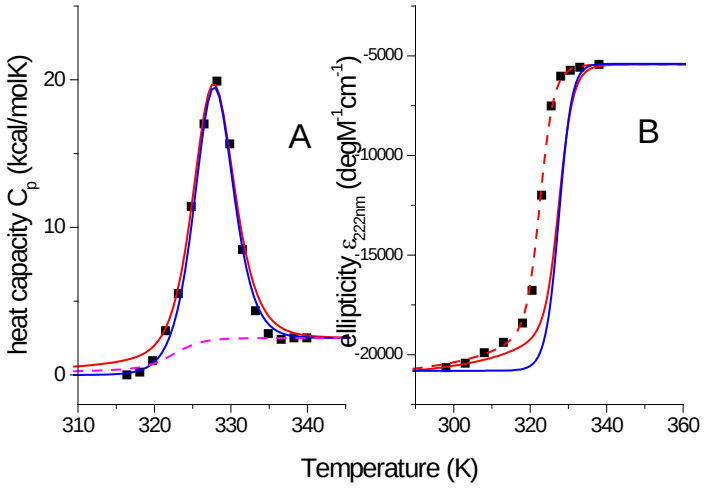

Figure S17. (A) The experimental DSC data (■)of wt T4 lysozyme are described almost equally well by the Zimm-Bragg theory (red solid line) and the 2-state model (blue solid line). The dashed magenta line shows the contribution of the heat capacity term $\Delta C^0_{p,NU}$ *to the transition, calculated with the Zimm-Bragg model. (B) The same parameters are used to calculate the CD transition. The 2-state model predicts a sharper CD transition than the Zimm-Bragg theory. Experimentally, the CD transition has its midpoint 5°C lower than the DSC transition. The dashed red line in figure (B) is identical to the solid red line but shifted by 5°C towards lower temperatures. Simulation parameters listed in table S10.*

Differential scanning calorimetry of pseudo wild-type T4 lysozyme (figure 2 in (Carra et al., 1996)) at pH 3.0 shows an unfolding transition between 318 K and 335K with a midpoint at T_0 = 327.5 K. As measured from figure 2 the molar heat capacity increases by $\Delta C^0_{p,NU} = 10.5\,\text{kJ mol}^{-1}\text{K}^{-1}$ = 2.508 kcal $\text{mol}^{-1}\text{K}^{-1}$ Numerical integration of the experimental data between 318K and 345K yields a total calorimetric heat of ΔH^0_{exp} = 662 kJ/mol = 158.1 kcal/mol. The best fit with the 2-state model is obtained with a conformational enthalpy of $\Delta H^0_{vH} = \Delta H^0_{NU}$ = 525 kJ/mol = 125.4 kcal/mol. (Table 1 in reference (Carra et al., 1996) lists T_0 = 327.1 K , $\Delta C^0_{p,NU}$ = 6.1 kJ $\text{mol}^{-1}\text{K}^{-1}$ = 1.459 kcal $\text{mol}^{-1}\text{K}^{-1}$ and $\Delta H^0_{vH} = \Delta H^0_{NU}$ = 533 kJ/mol = 127.5 kcal/mol). It should be noted that ΔH^0_{exp} includes both the conformational change and the contribution of the increase in heat capacity $\Delta C^0_{p,NU}$. The experimental DSC scan (figure 2) can be simulated with either the Zimm-Bragg theory or the 2-state model. The quality of the fit is almost perfect with the Zimm-Bragg theory and the calculated unfolding enthalpy of $\Delta H^0_{calc,ZB} = 658.9\text{kJ mol}^{-1} = 157.6\text{kcal mol}^{-1}$ agrees well with the experimental result. (If we assume that the unfolding requires 1.1 kcal/mol per segment, 143 segments participate in this process) The fit is less good for the 2-state model and the total unfolding enthalpy is only $\Delta H^0_{calc,2-state} = 595.6\text{kJ mol}^{-1} = 142.5\text{kcal mol}^{-1}$.

The enthalpy of the conformational change proper, ΔH^0_{NU}, can be calculated if the contribution of the molar heat capacity, that is, $\int_{318K}^{335K} \Delta C^0_{p,NU}\Theta_U(T)dT$, to the phase transition is subtracted. $\Theta_U(T)$ denotes the extent of unfolding ($0 \le \Theta_U(T) \le 1$). The integral can be estimated by either choosing a judicial baseline in figure 2 in (Carra et al., 1996) or simulating the unfolding transition with the Zimm-Bragg theory or the 2-state model. The contribution of the heat capacity change to the total unfolding enthalpy is $\Delta H^0_{Cp,NU}$ = 90.7 kJ/mol = 21.7 kcal/mol (Zimm-Bragg theory) or 79.5 kJ/mol=19.0 kcal/mol (2-state model). This leads to a

conformational enthalpy of 568 kJ/ mol=135.7 kcal/mol (Zimm-Bragg theory) or 525 kJ/ mol=125.4 kcal/mol (2-state model) .Table 1 (Carra et al., 1996) reports ΔH_{fit} =534 kJ/mol at pH 3.0.

The Zimm-Bragg theory and the 2-state model provide excellent descriptions of the differential scanning experiment and can therefore be used to predict the unfolding curve $\Theta_U(T)$ vs. T, measured with CD spectroscopy as shown in figure 5 of reference (Carra et al., 1996). The shape of the CD transition curve (steepness) is well reproduced by both models with the same parameters which were used to fit the calorimetric data. However, the spectroscopic transition is shifted by about 5°C towards lower temperatures compared to the DSC transition. A detailed comparison shows a clearly better fit of the CD spectroscopy data by the Zimm-Bragg theory than by the 2-state model.

The temperature difference between DSC and CD is not discussed in the original publication (Carra et al., 1996).

Table S17

Pseudo WT T4 lysozyme (164 aa, 19.0 kDa) pH 3.0(Carra et al., 1996)

	T_0, T_∞ [a]	T_{ini}-T_{end} [b]	$\Delta C^0_{p,NU}$ [c]	ΔH^0_{NU}, h [d]	ΔH^0_{total} [e]	N_{ZB} [f]	σ [g]
	K	K	kcal/molK	kcal/mol	kcal/mol		
DSC Experiment	327.5	318-335	2.508		158.2		
2-state model	327.6		2.508	125.4	144.4		
Zimm-Bragg theory	341.2		2.508	1.1	157.4	140	5×10^{-7}
CD Experiment	$T_{0,CD}$ [h]	ε_{222nm} [i]		f_{helix} [k]		ΔN_α [l]	
	K	$10^3 degM^{-1}cm^{-1}$					
	322.5	-20.65 – -5.43		0.6		100	

S18 Free energy of unfolding per peptide unit as a function of the width of the transition

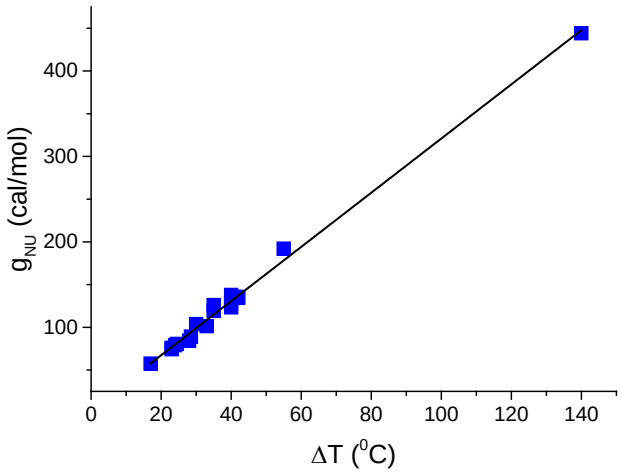

Figure S18.The free energy of unfolding per peptide unit, $\boldsymbol{g_{NU}}$*, is calculated with the Zimm-Bragg theory (see table 3 of manuscript). The width of the transition,* $\boldsymbol{\Delta T}$*, is taken from the experimental DSC scans (table 1 of manuscript). Linear regression leads to* $g_{NU}(cal/mol)=3.17\Delta T+3.9$ *(*$R^2=0.994$*).*

S 19 HPr protein

Nicholson,E.M., and Scholtz, J.M. (1996), *Biochemistry 35*, 11369-11378

"Conformational stability of the Escherichia coli HPr protein: Test of the Linear Extrapolation Method and a thermodynamic characterization of cold denaturation"

The Zimm-Bragg theory has not been applied yet to chemical protein denaturation. This is nevertheless possible by a simple modification of eqs (18)-(23). The temperature variable T must be replaced by the concentration of the denaturant c_D , the midpoint temperature T_∞ by the concentration c_{mid}, the concentration of denaturant at 50% unfolding, and the equilibrium constant is $s(c_D) = e^{-K_a(c_D - c_{mid})}$. The chemical unfolding is induced by binding of denaturant to protein sites with binding constant K_a (Makhatadze & Privalov, 1992). The nucleation parameter σ and the number of peptide units N involved in the transition retain their meaning.

HPr is a small globular protein (85 amino acids) with no disulfide bonds or other prosthetic groups (Jia et al., 1993). HPr serves as a phosphocarrier protein in the phosphoenolpyruvate-dependent carbohydrate transport system (PTS) in both Gram-negative and Gram-positive bacteria.

Using CD spectroscopy thermal unfolding was compared with chemical urea-denaturation(Nicholson & Scholtz, 1996). No DSC measurements were performed. The following data are therefore not included in tables 1-3 of the main manuscript.

At medium urea concentrations, both cold- and heat-denaturation could be observed. From the temperature difference between cold- and heat-denaturation the molar heat capacity $\Delta C^0_{p,NU}$ could be deduced and was $\Delta C^0_{p,NU}$ ~ 1.2kcal/molK at 20°C(Nicholson & Scholtz, 1996).

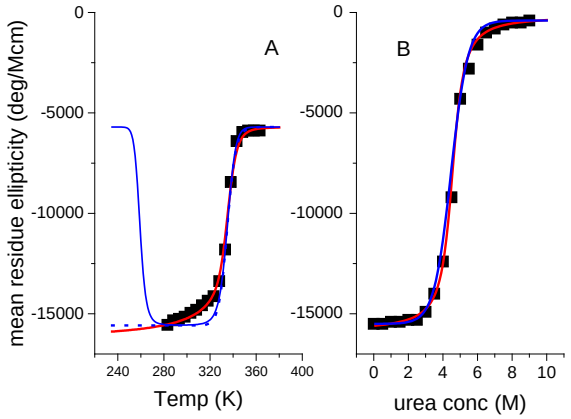

Figure S19. (A) Thermal and (B) urea-induced unfolding transitions of ecHPr as monitored by CD spectroscopy at 222 nm. (□)Experimental data taken from reference (Nicholson & Scholtz, 1996), figure 1. The thermal unfolding of ecHPr was in aqueous buffer only (10 mM K/P$_i$, pH 7.0). The urea denaturation curve was recorded in the same buffer, plus the indicated amount of urea (measurement at 15°C). The solid red line is the prediction of the Zimm-Bragg theory, the solid blue and dashed blue lines are those of the 2-state model. For the simulation parameters see text and table S19.

Thermal unfolding. The parameters of the Zimm-Bragg model are h = 1.1 kcal/mol, $\sigma = 1x10^{-5}$, N = 85, and T_{inf} = 351.5K. Panel A demonstrates that the Zimm-Bragg theory provides a better fit to the unfolding transition than the 2-state model. The curved baseline at the beginning of the transition is a consequence of the cooperativity. An empirical baseline correction is not necessary.

The 2-state model has the advantage that it predicts both heat- and cold-denaturation if $\Delta C^0_{p,NU} > 0$. In a CD spectroscopy experiment $\Delta C^0_{p,NU}$ can be determined from the temperature difference between cold- and heat-denaturation, but not from heat-denaturation alone. The solid blue line in panel A was calculated with ΔH^0_{NU} = 61.4 kcal/mol , T_0 = 335K and $\Delta C^0_{p,NU}$ = 1.30 kcal/molK. The midpoint of the cold-denaturation is then predicted at 248 K, in

agreement with (Nicholson & Scholtz, 1996). The dashed blue line was calculated with the same parameters, except $\Delta C^0_{p,NU}$ = 0 kcal/molK. The high-temperature transition is simulated equally well as before, but the cold denaturation is lost. This demonstrates that $\Delta C^0_{p,NU}$ has practically no influence on the shape of the high-temperature transition.

The heat-induced unfolding transition takes place between 320 K and 354 K (ΔT =34 K). Based on the 2-state model the unfolding enthalpy is calculated as $\Delta H^0_{calc,2-state}$ = 84.8 kcal/mol (including the contribution of the heat capacity $\Delta C^0_{p,NU}$). The unfolding enthalpy per peptide unit is thus ~1.0 kcal/mol.

The free energy of unfolding, ΔG^0_{NU}, calculated with the Zimm-Bragg theory is 9.4 kcal/mol, calculated with the 2-state model it is 6.4 kcal/mol.

Chemical denaturation. Figure S19B demonstrates that chemical denaturation with urea is slightly more efficient than thermal denaturation. Urea denaturation leads to a maximum mean residue ellipticity at 222 nm of -400 deg/Mcm, thermal denaturation to -5875 deg/Mcm. In terms of α-helix content the helix fraction is 0.18 in 6 M urea at 15 ^{0}C and 0.23 in buffer without urea at 90°C. However, the evaluation of the α-helix content at a single wavelength is not too reliable at low helix content (see main manuscript fig.2).

The solid blue line refers to the 2-state model calculated with

$$\Delta G(c_D) = \Delta G^0 - mc_D$$

and

$$K(c_D) = e^{\frac{-\Delta G(c_D)}{RT_0}}$$

(ΔG^0 = 5.41 kcal/mol; m = 1.21 kcal$M^{-1}$$mol^{-1}$, in agreement with table 1, 15^{0}C of reference(Nicholson & Scholtz, 1996)). No baseline correction was made.

The solid red line in panel B was calculated with the Zimm-Bragg theory using $\sigma = 4.0 \times 10^{-4}$, $K_a = 0.08\ M^{-1}$ and $c_{mid} = 4.55$ M. A urea binding constant of $K_a \sim 0.06\ M^{-1}$has been reported previously for 3 different proteins at 25°C (Makhatadze & Privalov, 1992).

In terms of the Zimm-Bragg theory the free energy of unfolding is determined by K_a and the steepness of the urea gradient between the beginning, c_N, and the end ,c_U, of the unfolding transition.

$$g_{urea} = RT \ln \frac{s(c_U)}{s(c_N)} = -RT[K_a(c_N - c_U)]$$

g_{urea} is the change in Gibbs free energy upon binding of 1 mole urea. For the example given above c_N = 4M and c_U= 6M, leading to g_{NU} = 0.137 kcal per mole bound urea. This is, in fact, a typical value for the free energy of unfolding obtained by completely different methods and described in detail in section 2.4 of the manuscript. If each amino acid residue of HPr binds 1 urea molecule, a total unfolding Gibbs free energy of 11.7 kcal/mol can be estimated.

Table S19

***Escherichia coli* HPr protein (85 aa, 9120 Da).CD spectroscopy only**

	T_0, T_∞ [a]	T_{ini}-T_{end} [b]	$\Delta C^0_{p,NU}$ [c]	ΔH^0_{NU}, h [d]	ΔH^0_{total} [e]	N_{ZB} [f]	σ [g]
	K	K	kcal/molK	kcal/mol	kcal/mol		
2-state model	335	320-354	1.3	61.4	84.8		
Zimm-Bragg theory	346.5			1.1	nd	85	6×10^{-5}
CD Experiment	$T_{0,CD}$ [h]	ε_{222nm} [i]	$\Delta C^0_{p,NU}$ [c]	f_{helix} [k]		ΔN_α [l]	
	K	$10^3 degM^{-1}cm^{-1}$	kcal/molK				
	335	-15.55 – -5.8	1.3	0.23		85	

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