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Conference on Structure and Dynamics in Soft Matter and Biomolecules: From Single Molecules to Ensembles

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Protein Stability

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PROTEIN STABILITY

Why are we interested in protein stability ?

1) Biotechnological implications:
Practical applications of proteins are often hampered by low stability.

2) Fundamental issues:
For instance: protein stabilization makes a good model problem to test and refine our knowledge about the structure-energetics relation in proteins (understanding the relation between structure and energetics is an essential requisite for rational protein design).

WHAT IS PROTEIN STABILITY ?

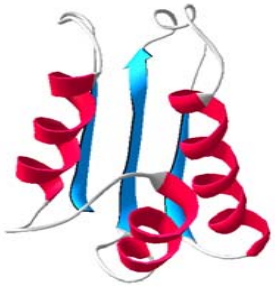
* High denaturation temperature

* It remains active during the time required for a given application (**kinetic stability**)

* It is stable under storage conditions for a long time: shelf life (**kinetic stability**).

* Comparatively high value of the unfolding free energy at room temperature (**thermodynamic stability**)

THERMODYNAMIC STABILITY vs. KINETIC STABILITY

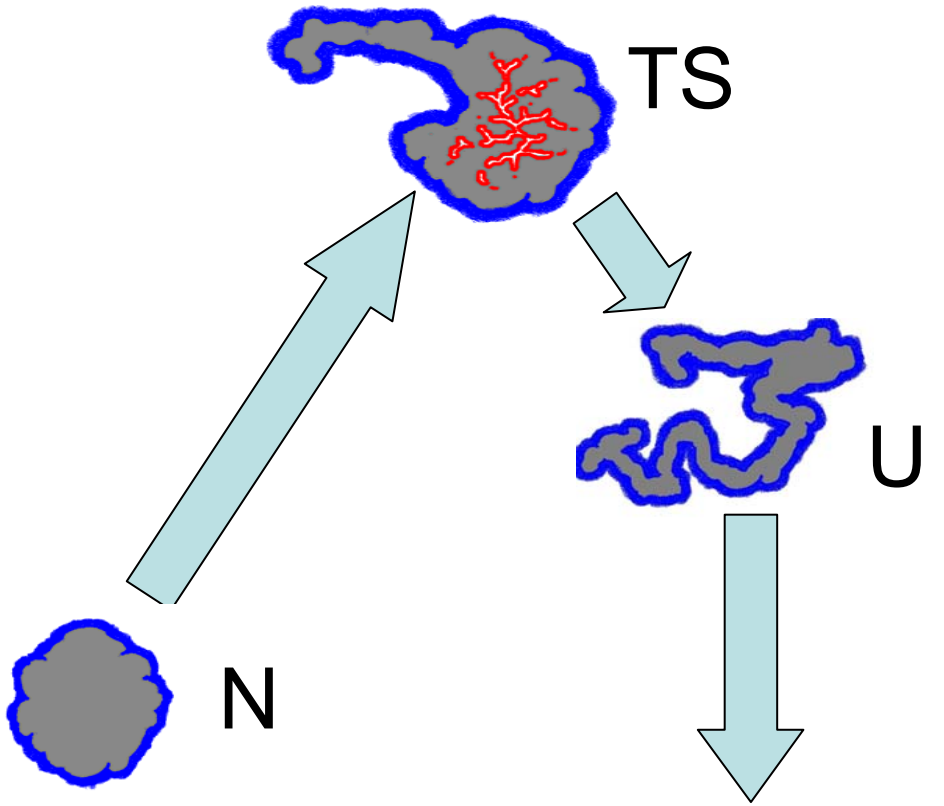


$\Delta G = G_U - G_N$

Native Unfolded

$K=\exp(-\Delta G/RT)$

$Xu=K/(1+K)$



IRREVERSIBLY DENATURED PROTEIN

$\tau=\tau_0\exp(\Delta G_{N\rightarrow TS}/RT)$

Time-scale for irreversible denaturation

Thermodynamic stability

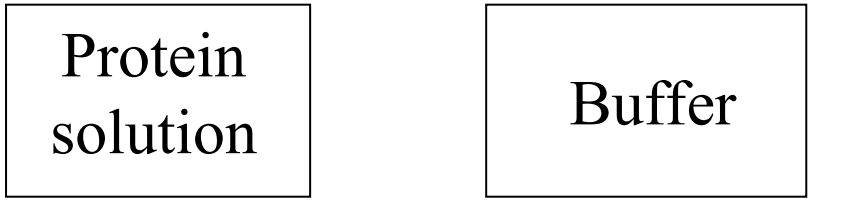
The protein stability curve
Structure-energetic relationships
Residual structure in protein denatured states
Deviations from two-state behavior: intermediate states
Deviations from two-state behavior: downhill folding

Kinetic stability

The role of transition-state structure
Alternative native or nearly-native states
Natural selection for kinetic stability

DIFFERENTIAL SCANNING CALORIMETRY

What is differential scanning calorimetry ?



Sample cell Reference cell

The sample and reference cells are heated at constant **scanning rate.**

When an endothermic process takes place in the sample cell (protein denaturation, for instance), its temperature lags behind that of the reference cell.

The feedback system of the calorimeter gives an extra power to the sample cell to eliminate the temperature difference. That extra power is proportional to the heat capacity of the protein solution (taking the buffer as reference)

What is heat capacity ?

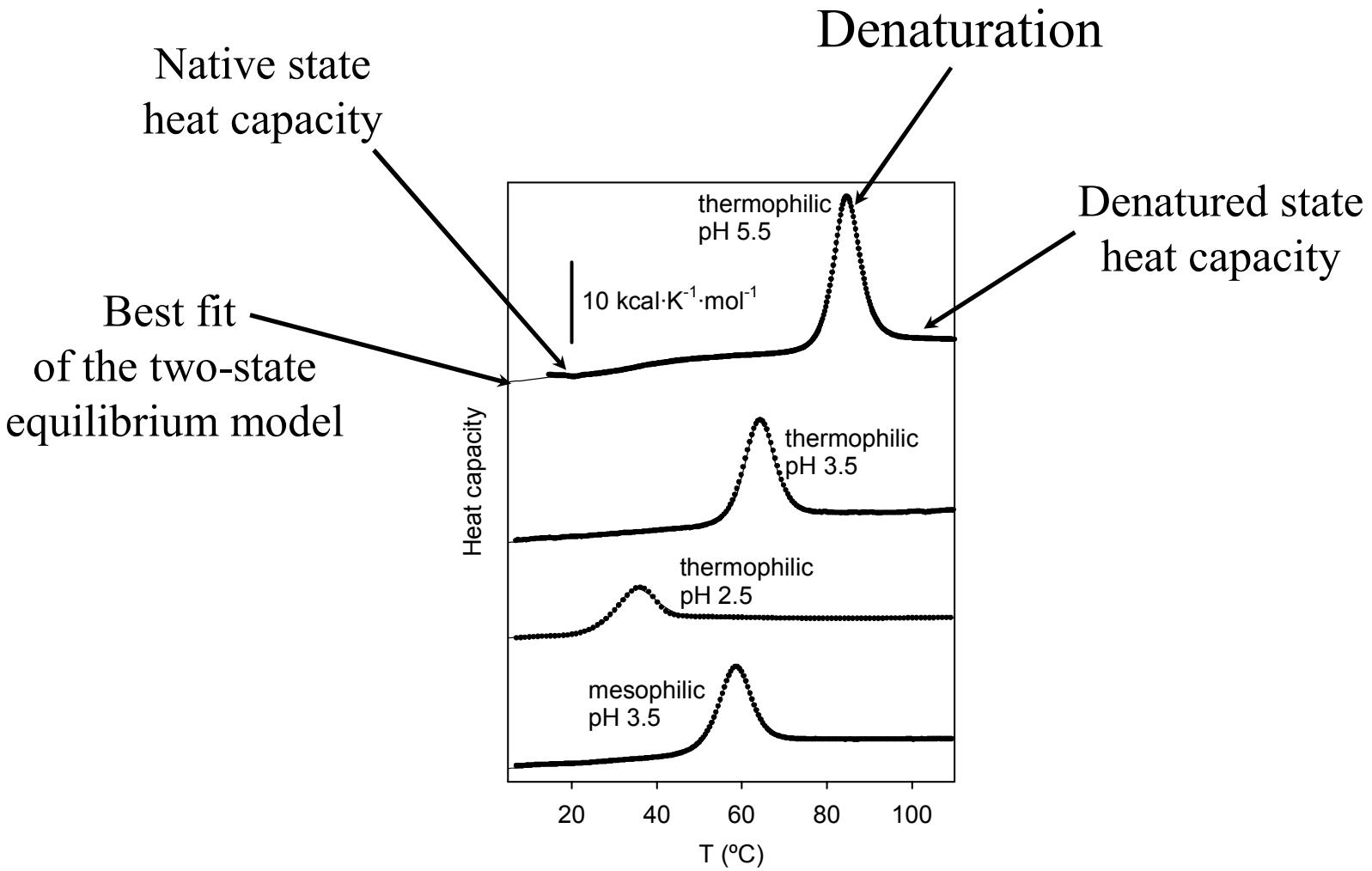
$$C_P = \frac{dH}{dT}$$

H ≡ Enthalpy ≅ Energy

Heat capacity is a measure of the system efficiency to store energy

Liquid water has a high heat capacity because of an efficient energy storage mechanism: breakage of hydrogen bonds.

Protein denaturation processes gives rise to “peaks” in DSC thermograms, because the denaturated protein has higher energy and denaturation provides an energy storage mechnism.



Representative examples of DSC thermograms:
Mesophilic and thermophilic ribonuclease H
Guzman-Casado, Parody-Morreale, Robic, Marqusee, Sanchez-Ruiz (2003)
J. Mol. Biol. 329:731-743.

The two-state equilibrium model

Only two macrostates of the protein are significantly populated: the native state (N) and the unfolded state (U).

$$N \Leftrightarrow U$$

The relative amounts of N and U are determined by an equilibrium constant that changes with temperature.

$$K = \frac{[U]}{[N]} = \exp\left(-\Delta G / RT\right)$$

$$\Delta G = \Delta H - T \cdot \Delta S$$

$$\frac{d\Delta H}{dT} = \Delta C_P$$

$$\frac{d\Delta S}{dT} = \frac{\Delta C_P}{T}$$

The average heat capacity of the states involved in the transition

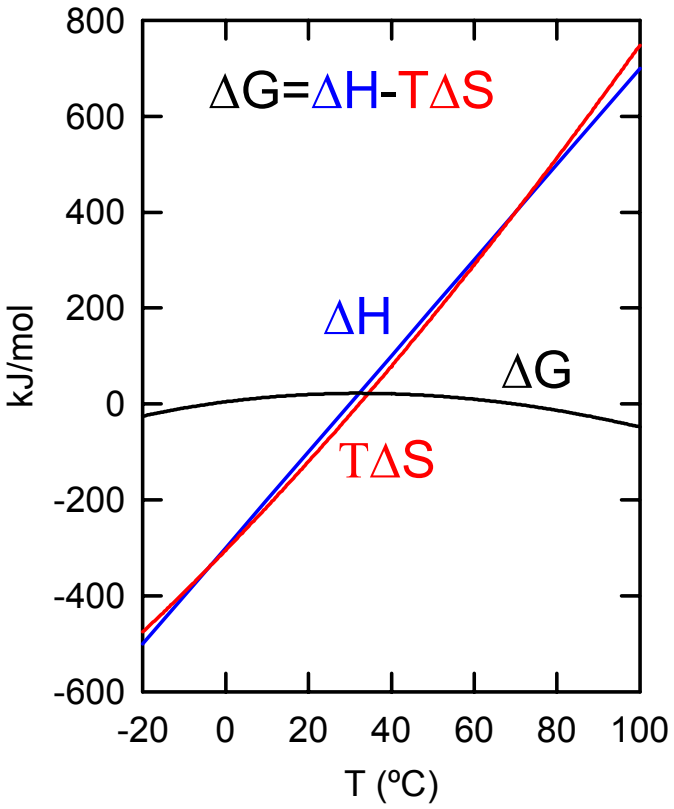
The temperature-induced shift in the populations of states

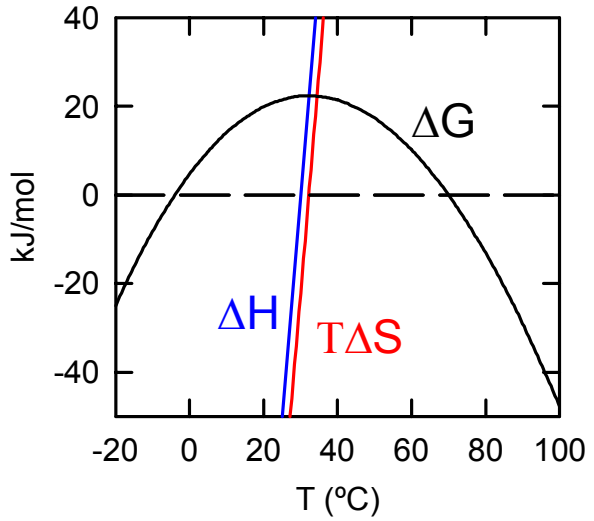
$$C_P = C_P(N) + \frac{K}{1+K}\Delta C_P + \frac{\Delta H^2}{RT^2}\frac{K}{(1+K)^2}$$

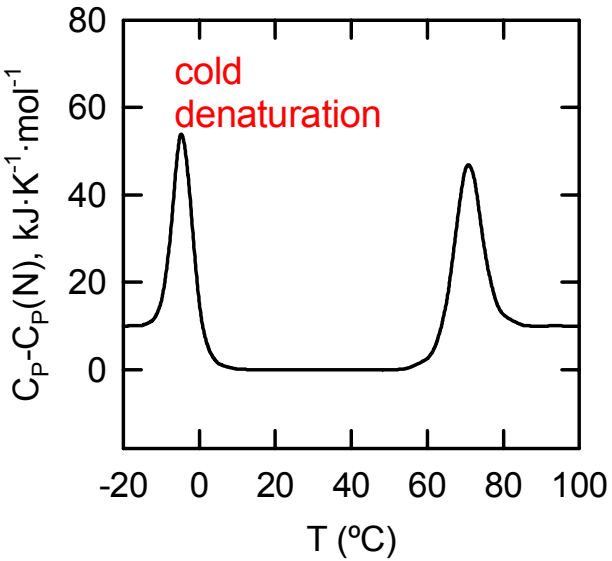
The protein stability curve

$\Delta H(T)=\Delta H(T_m)+\Delta C_P\cdot(T-T_m)$

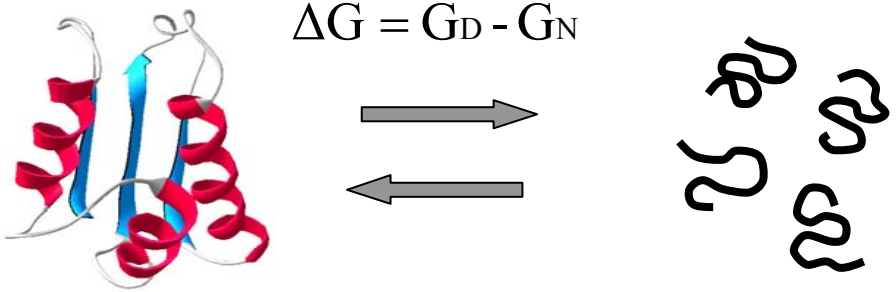
$\Delta S(T)=\Delta H(T_m)/T_m+\Delta C_P\cdot\ln(T/T_m)$







Structure-energetics relationships



ΔC_P: interactions with the solvent of residues exposed upon unfolding.

ΔH: breaking of internal interactions (van der Waals interactions, hydrogen bonds…) and interactions with the solvent of residues exposed upon unfolding.

ΔS: conformational entropy change (backbone and side-chain contributions) and Interactions with the solvent of residues exposed upon unfolding.

Parametrization in terms of unfolding changes in accessible surface area!

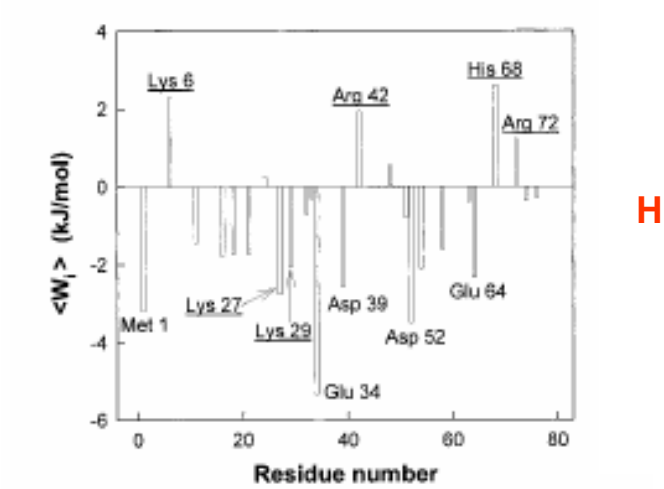
Luque and Freire (1998) Methods Enzymol. 295:100-127.
Robertson and Murphy (1997) Chem. Rev. 97:1251-1267.

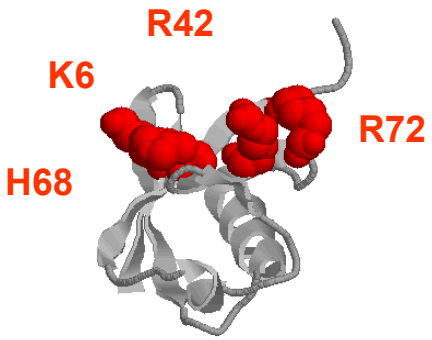
Table 5. Results of Regression Analysis of Thermodynamics of Protein Unfolding

thermodynamic parameter	regression variables	regressed values	R^2
ΔC_p	N_{res}	58 ± 1 J K^{-1} (mol res)$^{-1}$	0.859
ΔC_p	ΔA_{tot}	0.61 ± 0.02 J K^{-1} (mol Å^2)$^{-1}$	0.856
ΔC_p	ΔA_{ap}	0.66 ± 0.21 J K^{-1} (mol Å^2)$^{-1}$	0.856
	ΔA_{pol}	0.52 ± 0.32 J K^{-1} (mol Å^2)$^{-1}$	
ΔH (60 °C)	N_{res}	2.92 ± 0.08 kJ (mol res)$^{-1}$	0.766
ΔH (60 °C)	ΔA_{tot}	30.2 ± 0.9 J (mol Å^2)$^{-1}$	0.735
ΔH (60 °C)	ΔA_{ap}	-8 ± 11 J (mol Å^2)$^{-1}$	0.775
	ΔA_{pol}	86 ± 17 J (mol Å^2)$^{-1}$	
ΔH (100 °C)	N_{res}	5.28 ± 0.09 kJ (mol res)$^{-1}$	0.918
$\Delta S°$ (60 °C)	N_{res}	8.8 ± 0.3 J K^{-1} (mol res)$^{-1}$	0.744
$\Delta S°$ (60 °C)	ΔA_{tot}	0.091 ± 0.003 J K^{-1} (mol Å^2)$^{-1}$	0.716
$\Delta S°$ (60 °C)	ΔA_{ap}	-0.03 ± 0.04 J K^{-1} (mol Å^2)$^{-1}$	0.757
	ΔA_{pol}	0.27 ± 0.06 J K^{-1} (mol Å^2)$^{-1}$	
$\Delta S°$ (60 °C)	N_{res}	9.2 ± 4.6 J K^{-1} (mol res)$^{-1}$	0.771
	ΔA_{ap}	-0.11 ± 0.05 J K^{-1} (mol Å^2)$^{-1}$	
	ΔA_{pol}	0.15 ± 0.08 J K^{-1} (mol Å^2)$^{-1}$	
$\Delta S°$ (112 °C)	N_{res}	17.3 ± 0.3 J K^{-1} (mol res)$^{-1}$	0.919

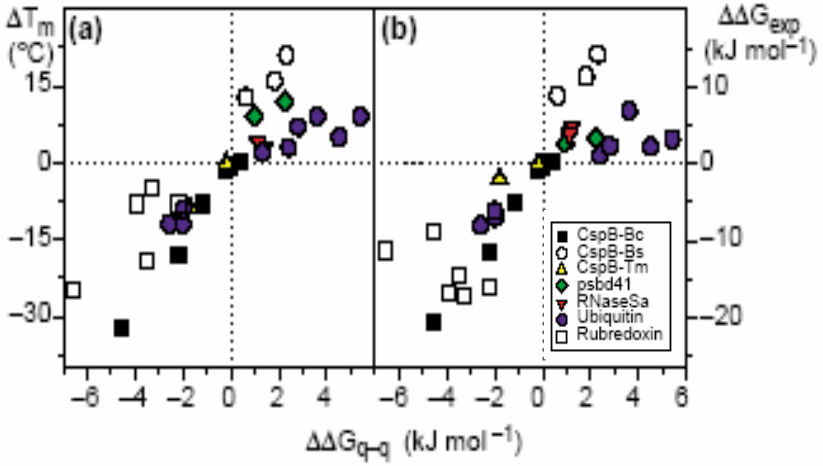
The good news…

Proteins can be stabilized through the rational desing of "minor" energetic contributions!

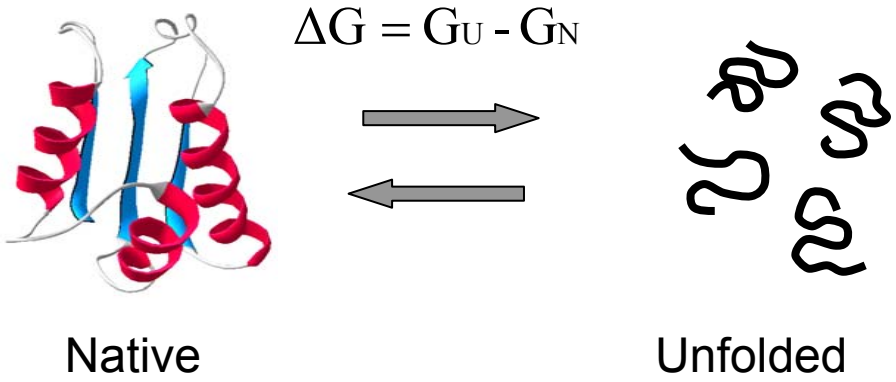




Human Ubiquitin

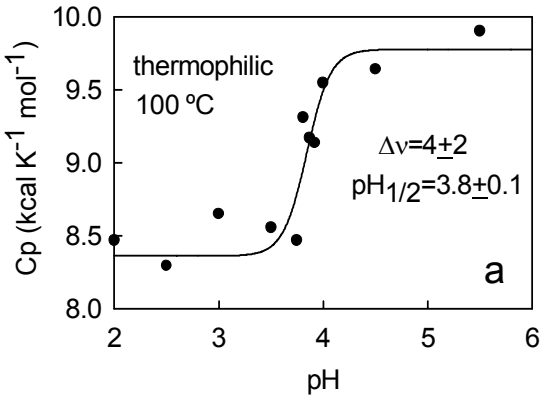


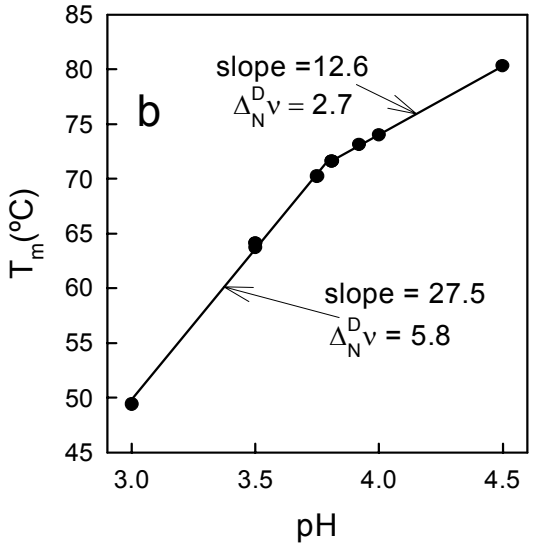
•Sanchez-Ruiz & Makhatadze, ***TRENDS in Biotechnology***, *Vol.19, No.4, 132-35 (*2001)
•Loladze, Ibarra-Molero, Sanchez-Ruiz & Makhatadze, ***Biochemistry***, *Vol.38, No.50, 16419-23 (*1999)
•Ibarra-Molero, Loladze, Makhatadze & Sanchez-Ruiz, ***Biochemistry***, *Vol.38, No.25, 8138-49 (*1999)

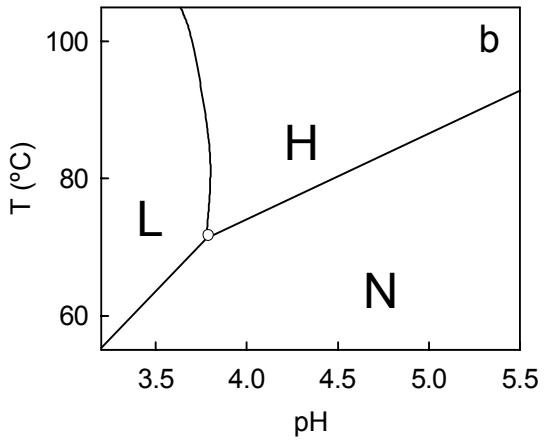


ISSUE: what is the unfolded state?

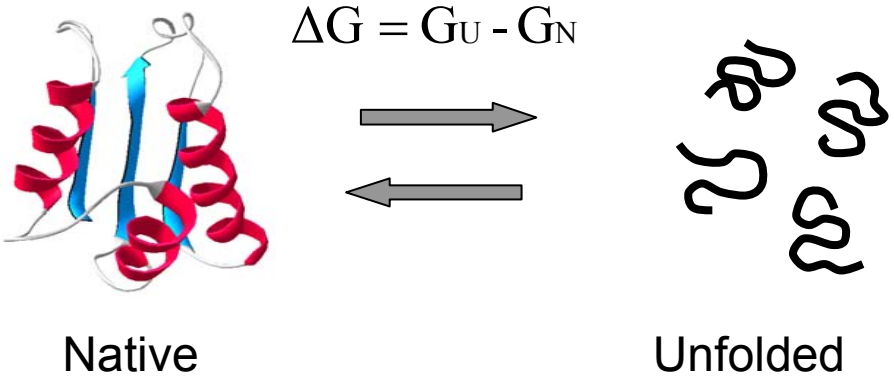
Guzman-Casado, Parody-Morreale, Robic, Marqusee, Sanchez-Ruiz (2003)
Energetic evidence for formation of a pH-dependent hydrophobic cluster in the denatured state of *Thermus thermophilus* ribonuclease H.
J. Mol. Biol. 329, 731-743.







Recent experimental studies suggest the existence of long-range interactions and cooperative processes in protein denatured states:
Shortle & Ackerman (2001) Science 293, 487-489.
Klein-Seetharaman et al. (2002) Science 295, 1719-1722.



ISSUE: what if there are intermediate states?

Undistorted DSC data contain all relevant information about equilibrium protein folding/unfolding mechanism

Freire & Biltonen (1978) *Biopolymers* **17,** 463-479

$$I_1\,(N) \Leftrightarrow I_2 \Leftrightarrow I_3 \Leftrightarrow \ldots\ldots \Leftrightarrow I_{n-1} \Leftrightarrow I_n\,(D)$$

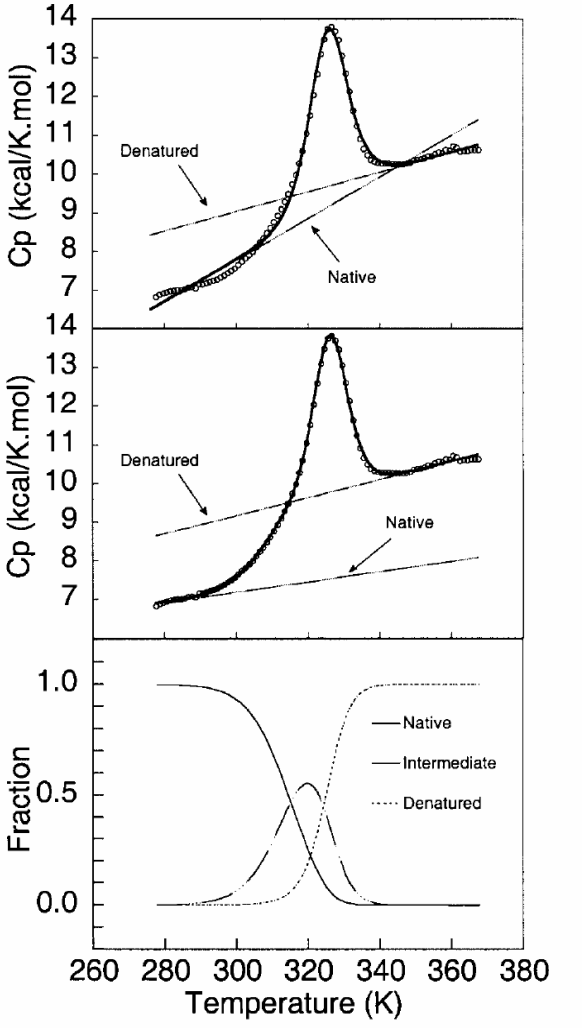
Partition function = sum of statistical weights

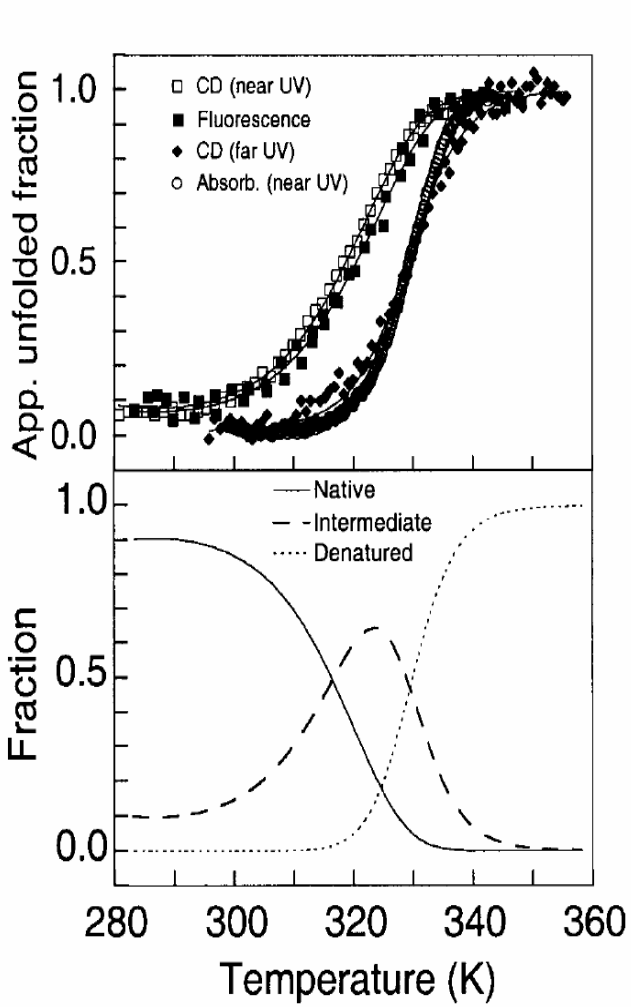
$$I_i \qquad \omega_i = \frac{[I_i]}{[I_1]} = K_i = \exp\left(-\Delta G_i / RT\right)$$

$$Q = \sum_i \omega_i = \sum_i \exp\left(-\Delta G_i / RT\right)$$

$$Q(T) = \exp\left\{\int_{T_0}^{T} 1/RT^{\,2}\left[\int_{T_0}^{T} C_P^{ex}\cdot dT\right]\cdot dT\right\}$$

Irun, Garcia-Mira, Sanchez-Ruiz, Sancho (2001). Native hydrogen bonds in a molten globule: the apoflavodoxin thermal intermediate.
J. Mol. Biol. 306, 877-888.





doi:10.1016/j.jmb.2004.09.047 J. Mol. Biol. (2004) 344, 223–237

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Do Proteins Always Benefit from a Stability Increase? Relevant and Residual Stabilisation in a Three-state Protein by Charge Optimisation

Luis A. Campos[1,2], Maria M. Garcia-Mira[1,3], Raquel Godoy-Ruiz[1,3] Jose M. Sanchez-Ruiz[1,3]* and Javier Sancho[1,2]*

Table 2. Relevant ($\Delta\Delta G_{N-I}$) and residual ($\Delta\Delta G_{I-D}$) conformational stabilisation in charge reversal mutant apoflavodoxins, relative to wild-type, as determined from three-state thermal denaturation global analysis

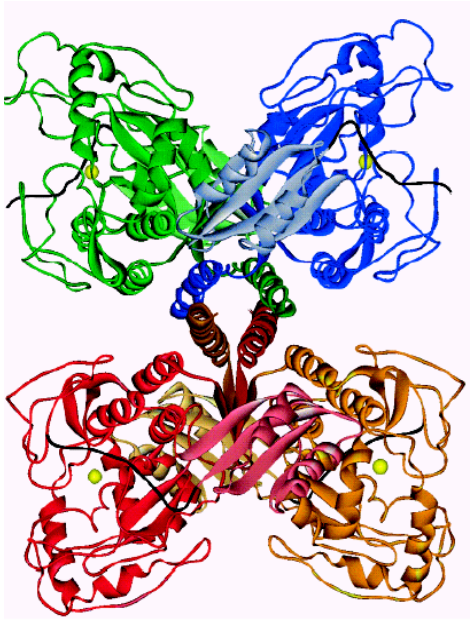
Protein	$T_{m1(NI)}$ (K)	$\Delta H_{1(NI)}$ (kcal mol^{-1})	$T_{m2(ID)}$ (K)	$\Delta H_{2(ID)}$ (kcal mol^{-1})	$\Delta\Delta G_{NI}$[a] (kcal mol^{-1})	$\Delta\Delta G_{ID}$[a] (kcal mol^{-1})	$\Delta\Delta G_{ND}$[b] (kcal mol^{-1})
Wt	317.3±0.2	33.9±0.1	329.0±0.1	52.7±0.6	–	–	
E20K	315.8±0.4	30.2±0.3	332.8±0.1	66.1±1.3	+0.14±0.05	−1.20±0.08	−1.06±0.09
E40K	317.9±0.4	33.5±0.1	333.5±0.1	62.5±1.1	−0.06±0.05	−1.16±0.07	−1.22±0.09
E61K	320.9±0.1	41.0±0.9	331.7±0.2	38.0±1.0	−0.46±0.03	+0.22±0.07	−0.24±0.07
D65K	315.2±0.4	30.4±0.1	329.2±0.1	54.9±0.9	+0.20±0.05	−0.11±0.06	+0.09±0.08
E72K	318.5±0.3	33.2±0.2	336.2±0.1	57.8±1.0	−0.13±0.04	−1.38±0.07	−1.50±0.08
D75K	317.4±0.2	34.2±0.1	334.2±0.1	59.6±0.8	−0.01±0.04	−1.14±0.06	−1.15±0.07
D126K	321.9±0.2	40.6±0.2	330.8±0.1	45.1±1.3	−0.58±0.04	+0.03±0.07	−0.55±0.08
D150K	317.2±1.1	30.4±0.7	329.6±0.4	44.7±1.2	+0.01±0.11	+0.21±0.08	+0.22±0.14

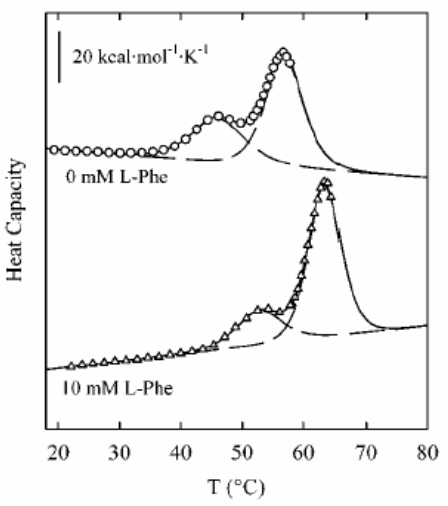
Thórolfsson, Ibarra-Molero, Fojan, Petersen, Sanchez-Ruiz, Martinez (2002). L-Phenylalanine binding and domain organization in human phenylalanine hydroxylase: a differential scanning calorimetry study. Biochemistry 41, 7573-7585

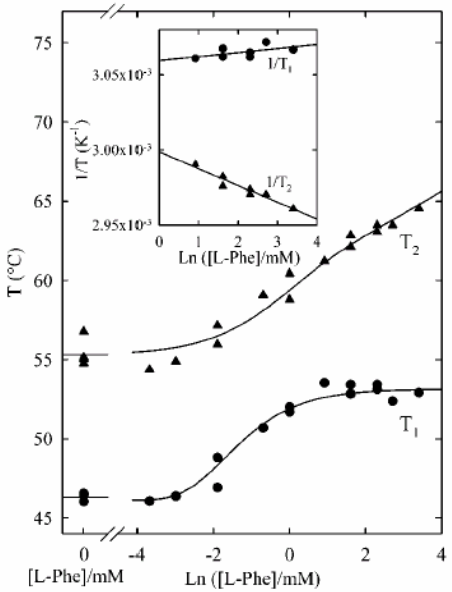
$$\exp\left[-\frac{\Delta H}{R}\left(\frac{1}{T_C}-\frac{1}{T_C^0}\right)\right]=\frac{P_A}{P_B}$$

T_C, characteristic temperature of the transition

P_A and P_B, binding polynomials for states A and B







There are no binding sites for L-Phe in the regulatory domains!

BIG ISSUE: What if the description in terms of well-defined states is not appropriate ?

Two-state model:

Native (N) $\rightleftharpoons$ Unfolded (U)

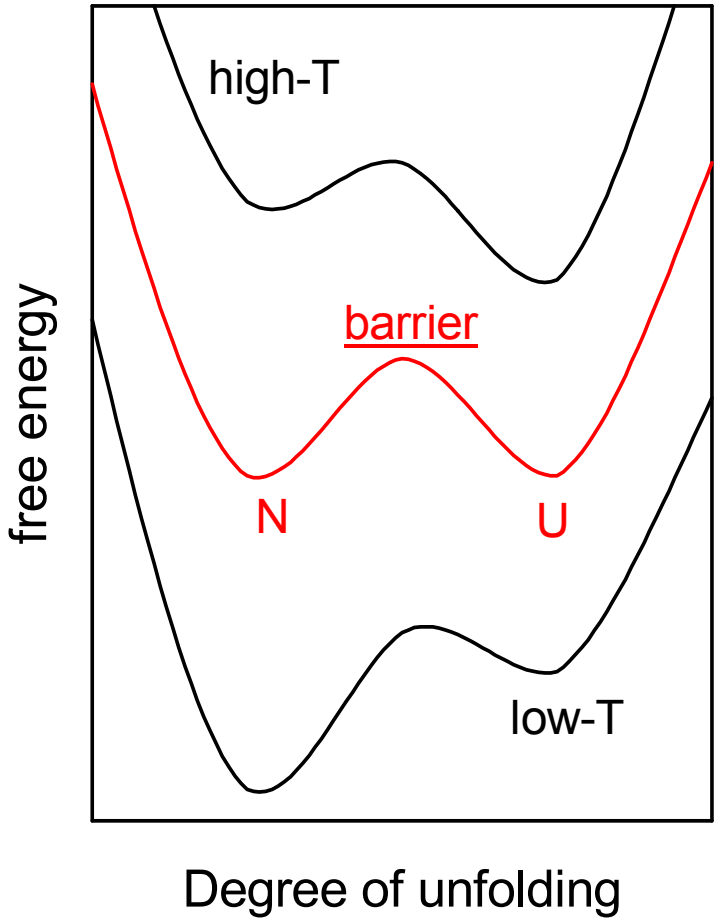
Multi-state equilibrium:

$I_0(N) \leftrightarrow I_1 \leftrightarrow I_2 \ldots\ldots\ldots I_{n-2} \leftrightarrow I_{n-1} \leftrightarrow I_n(U)$

In a sense, these are chemical models.

But, protein folding is not a chemical reaction

Two macrostate scenario:



High free energy for microstates of Intermediate degree of unfolding.

Thermodynamic barrier.

Cooperative unfolding.

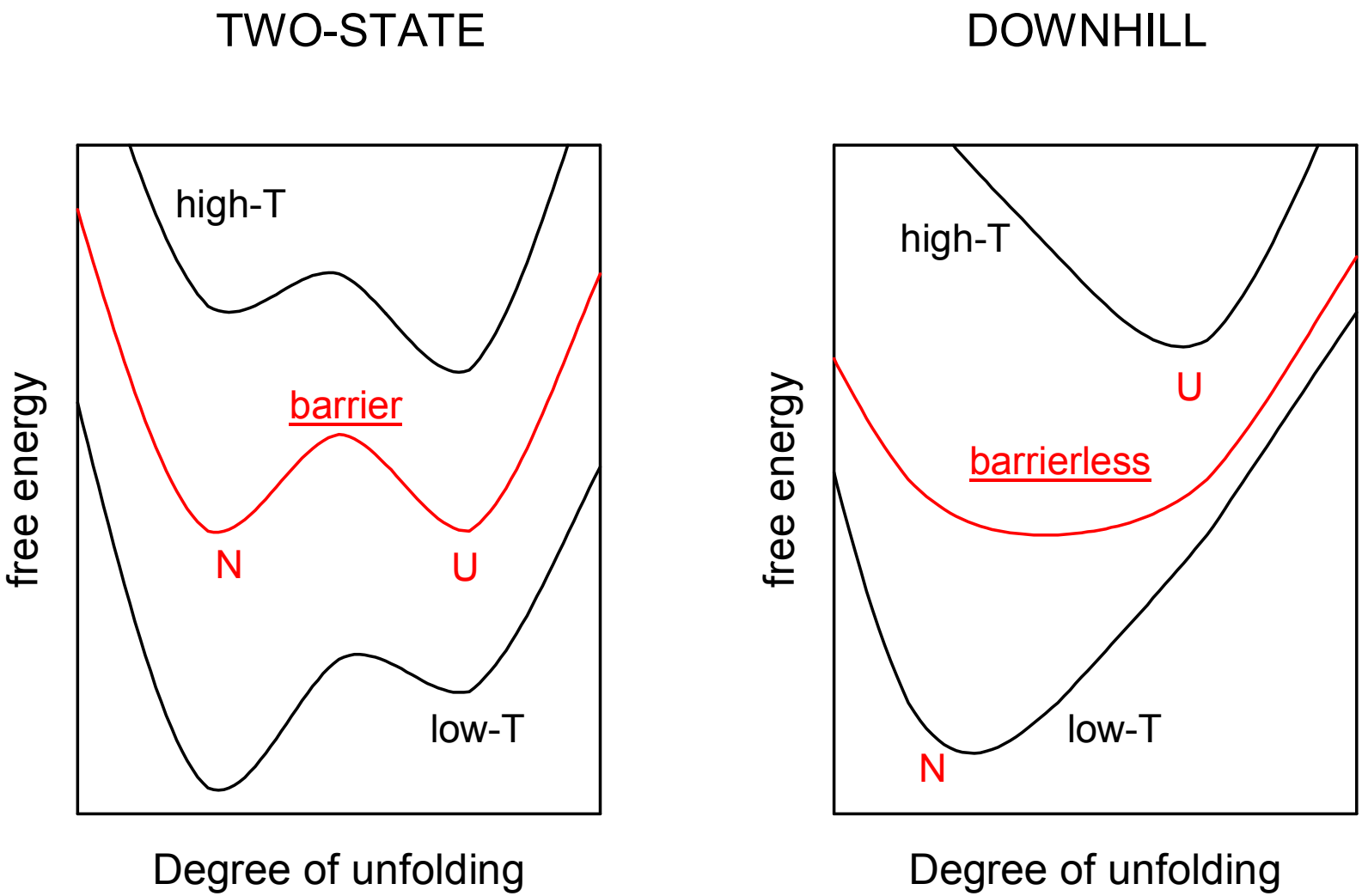
Kinetic analysis suggest that folding barriers for many natural proteins are small in the chemical sense [Kubelka, Hofrichter, Eaton (2004) *Current Opinion in Structural Biology* 14, 76-88].

Computer designed proteins fold faster (lower barrier) than their natural counterparts, although no selection for folding efficiency was included in the design strategy [Scalley-Kim, Baker (2004) *Journal of Molecular Biology* 338, 573-583].

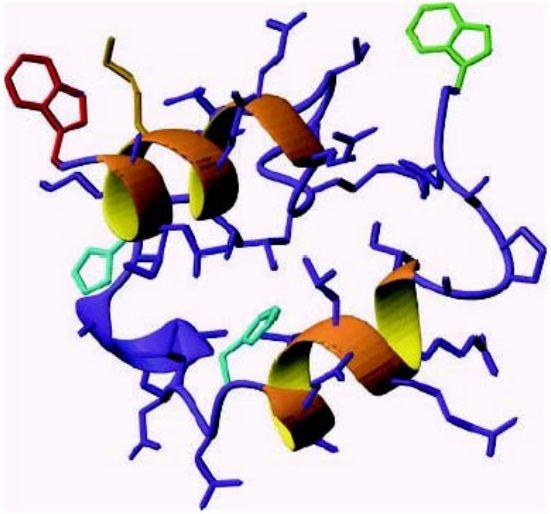
Perhaps, folding barriers are not an intrinsic feature of protein folding and must not be taken for granted (e.g., the Corex program of Freire and cols.)

If there is **no barrier**, we have:

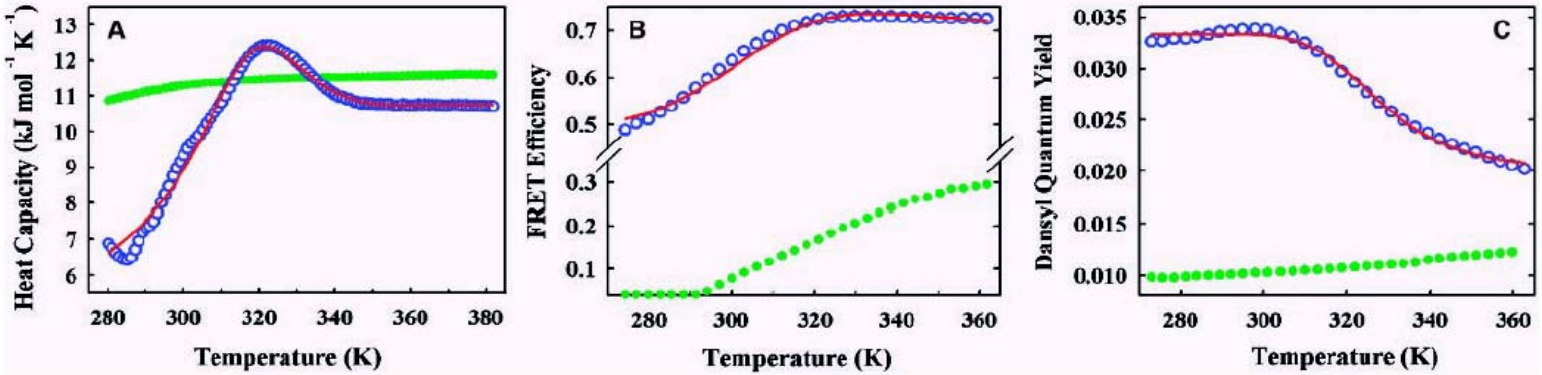
Barrierless, single-state, continuous, gradual, non-cooperative,
DOWNHILL FOLDING


TWO-STATE
high-T
barrier
N
U
low-T
free energy
Degree of unfolding
DOWNHILL
high-T
U
barrierless
N
low-T
free energy
Degree of unfolding

Garcia-Mira, Sadqi, Fischer, Sanchez-Ruiz, Muñoz (2002). Experimental identification of downhill folding.
Science 298, 2191-2195.



BBL



Adapting classical Landau theory to protein folding:

We describe the protein as an ensemble of “enthalpy microstates” and write the partition function in terms of a density of states:

$$Q = \int \rho(H) \cdot \exp\left(-H/RT\right) \cdot dH$$

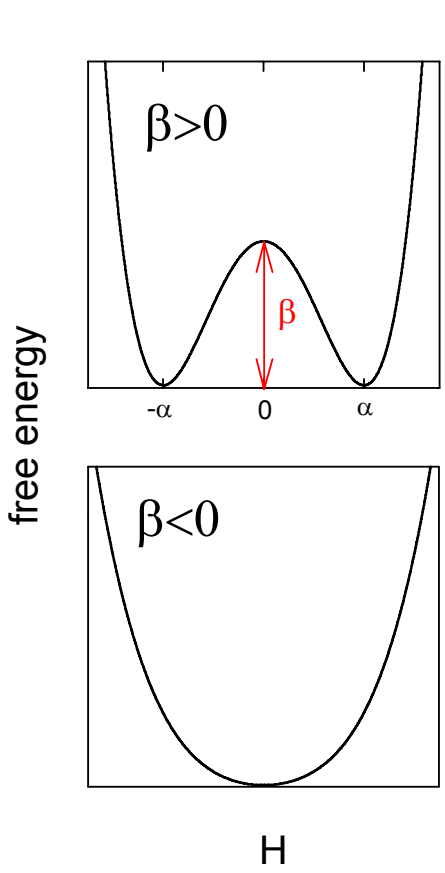
H is an ethalpy scale defined by native baseline substraction. We expect this enthalpy scale to be reasonably close to a true structural scale and we use H as the order parameter in a Landau-style expansion of the free energy at a characteristic temperature (T_0):

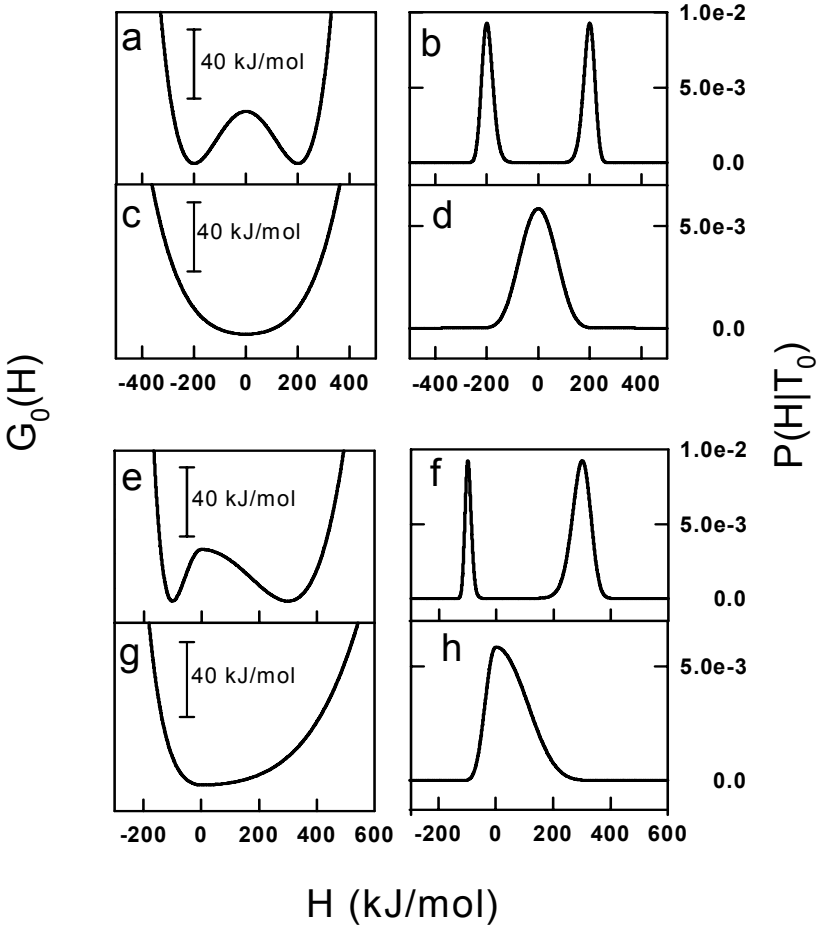
$$G_0(H) = -2\beta\left(\frac{H}{\alpha}\right)^2 + |\beta|\left(\frac{H}{\alpha}\right)^4$$

The sign of β determines whether there is a barrier or not!

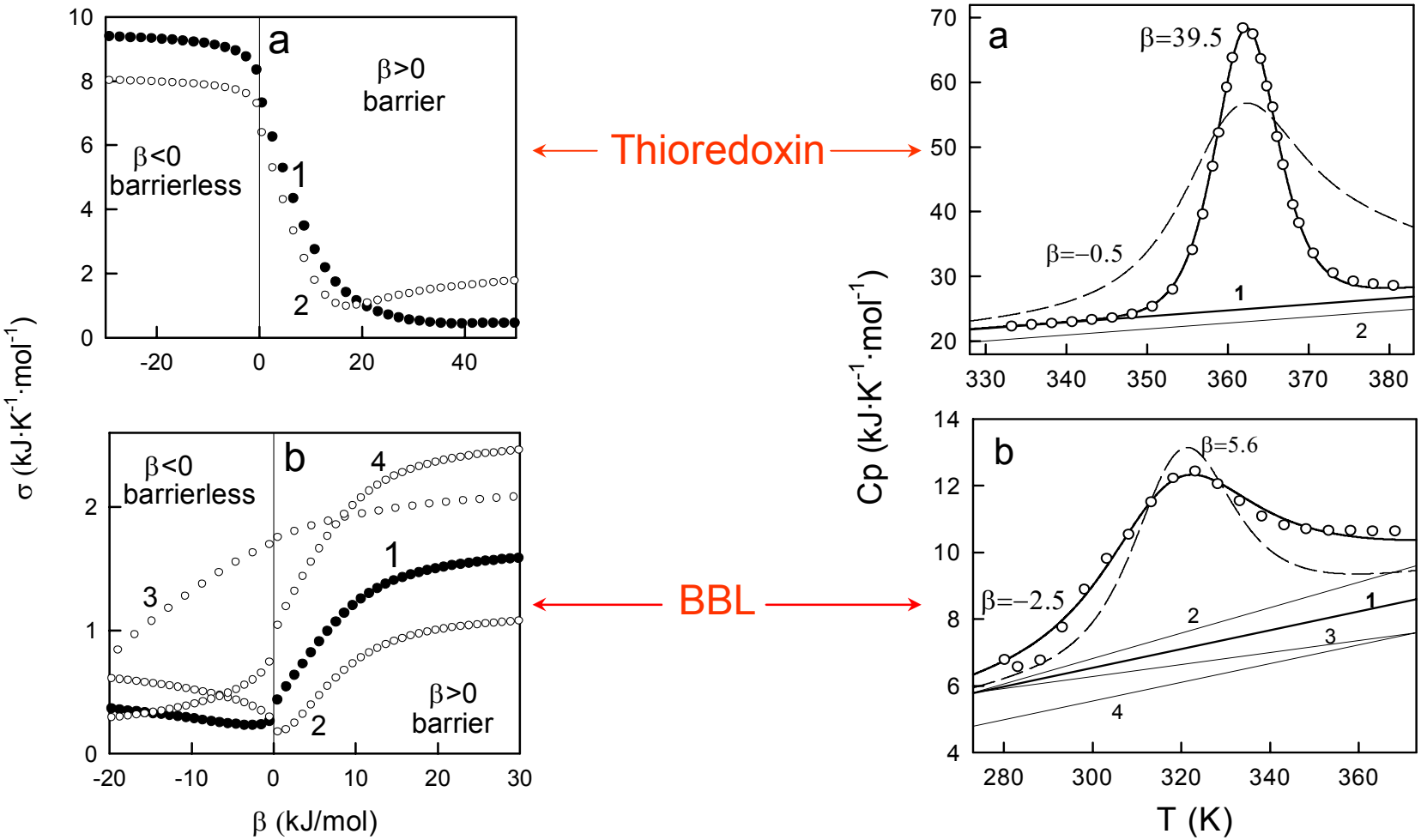
If β>0, its value gives the height of the barrier.

The Landau free-energy functional leads to two-macrostate or global-downhill folding scenarios depending on the value of β



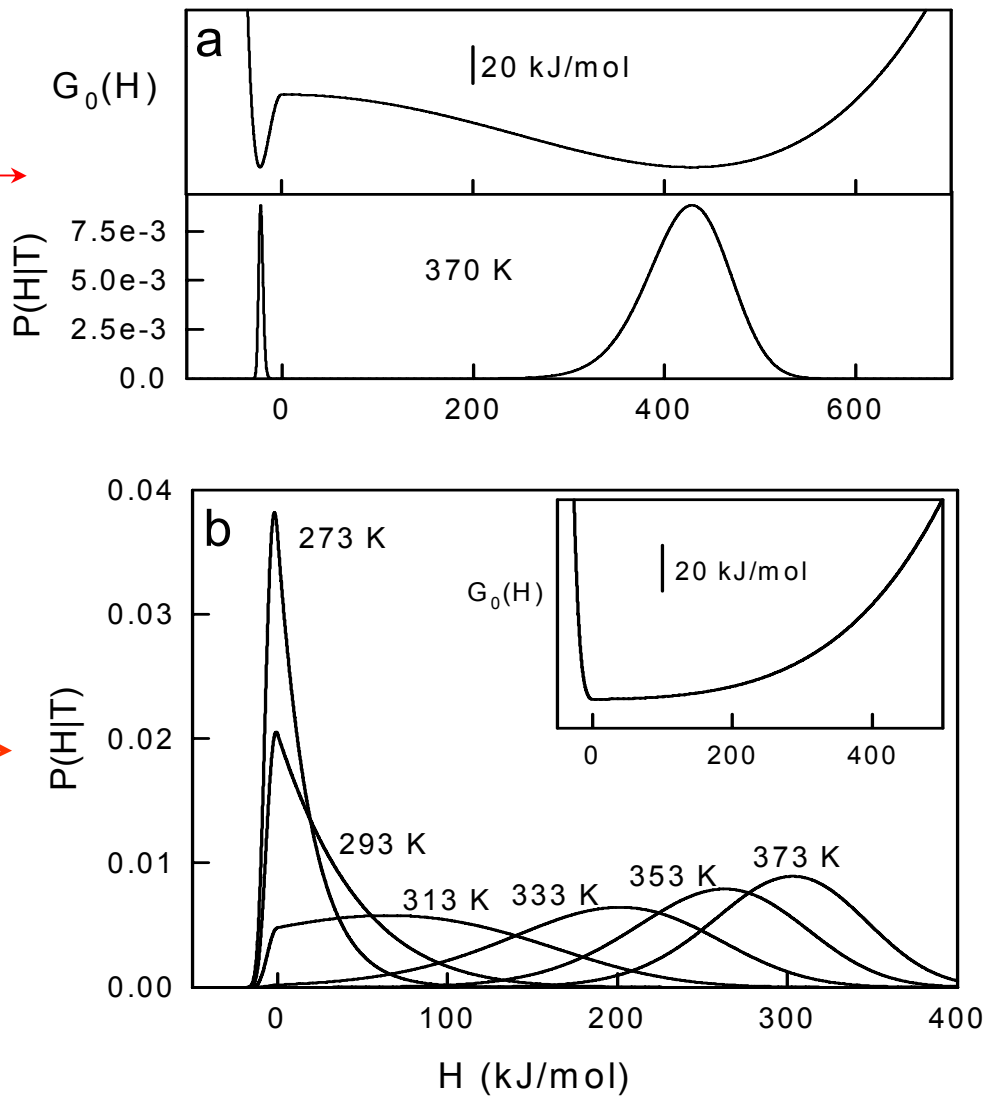


Barrier heights can be determined from the fitting of the model to the experimental DSC profiles



Thioredoxin →

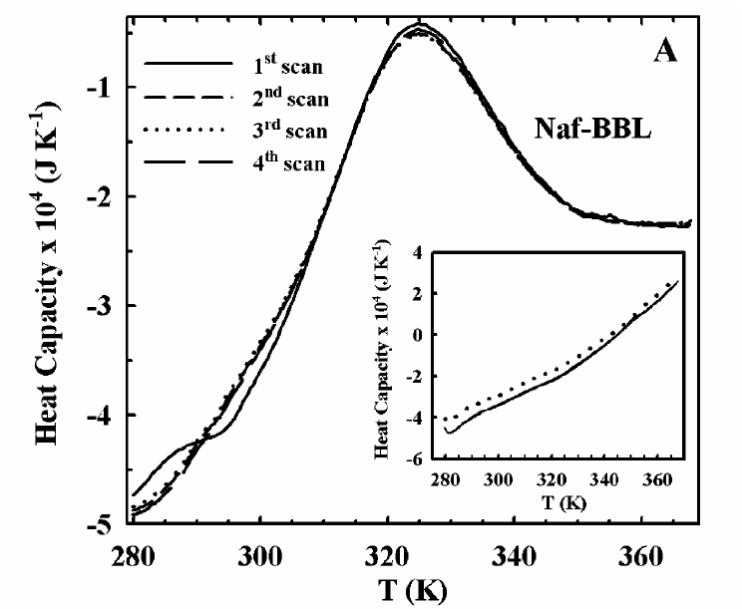
BBL →



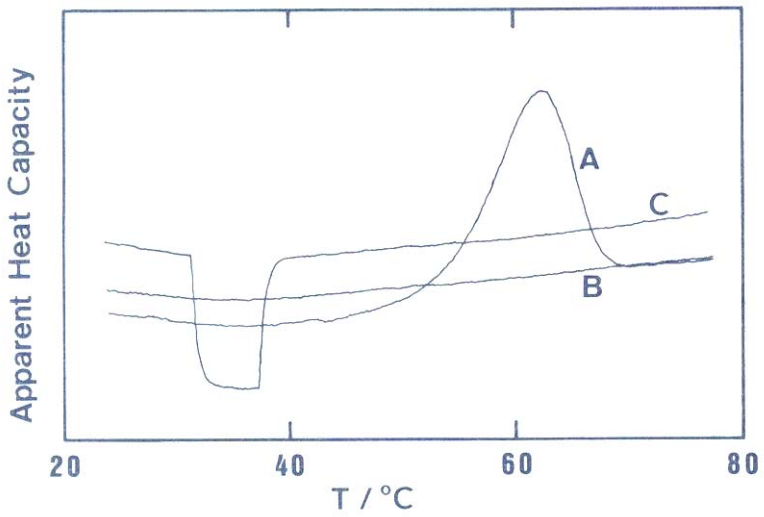
Muñoz, Sanchez-Ruiz (2004) PNAS 101:17646-17651

AN EVEN BIGGER ISSUE: How do we know that we can apply equilibrium thermodynamics ?

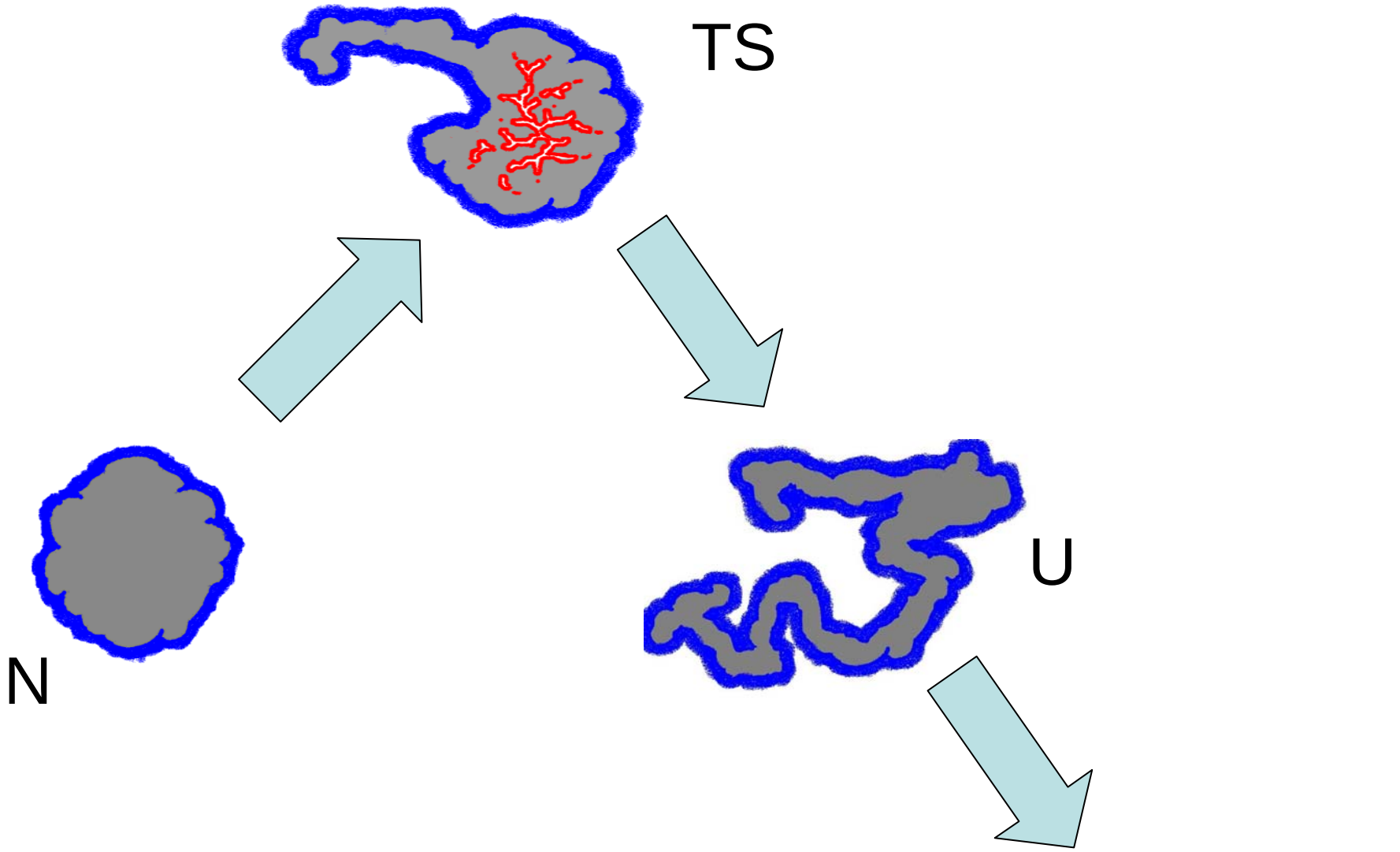
Calorimetric reversibility!



Calorimetrically Reversible



Calorimetrically Irreversible

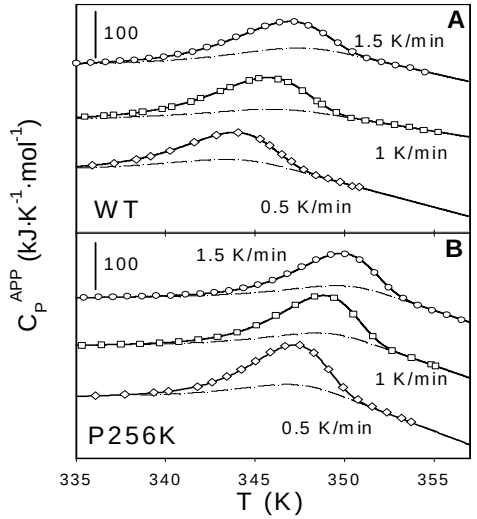


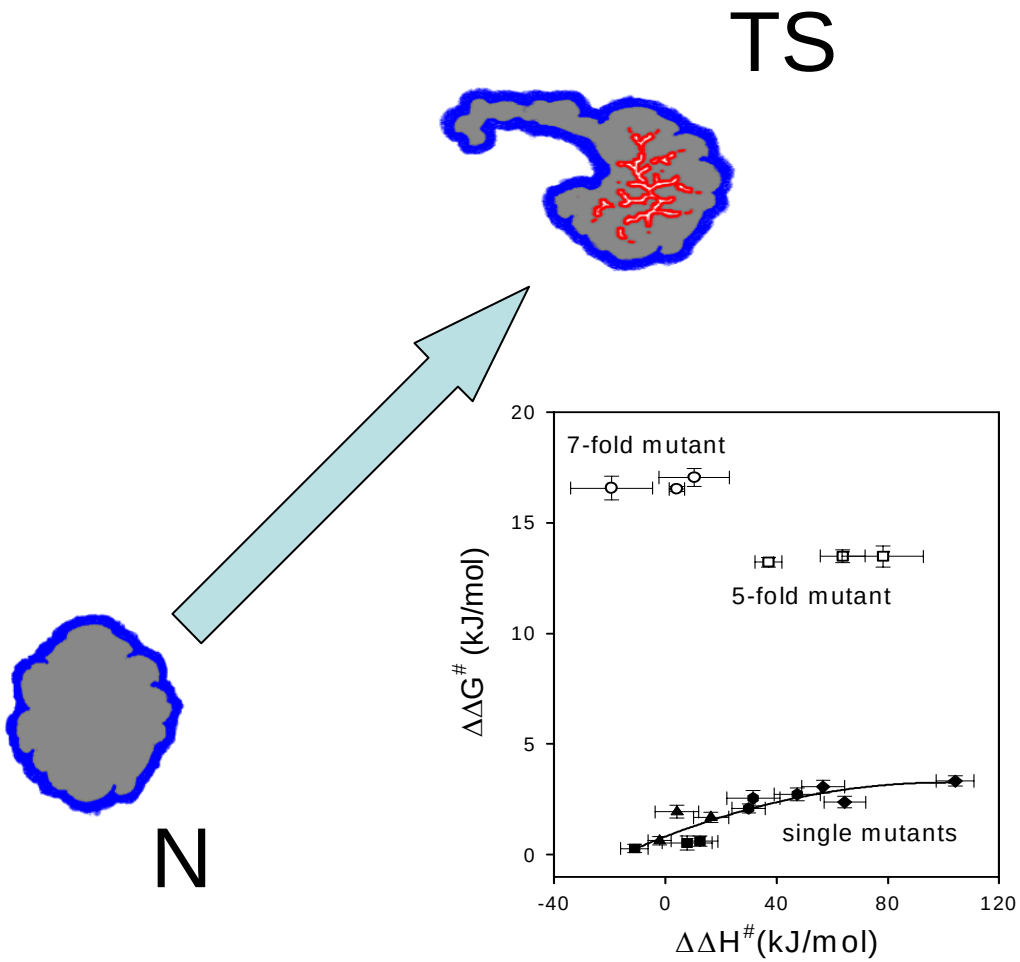
Lumry-Eyring model

IRREVERSIBLY DENATURED PROTEIN

Kinetic stability is important in biotechnological applications

Thermal denaturation of lipase is irreversible, kinetically-controlled and conforms to the two-state irreversible model (N→F)

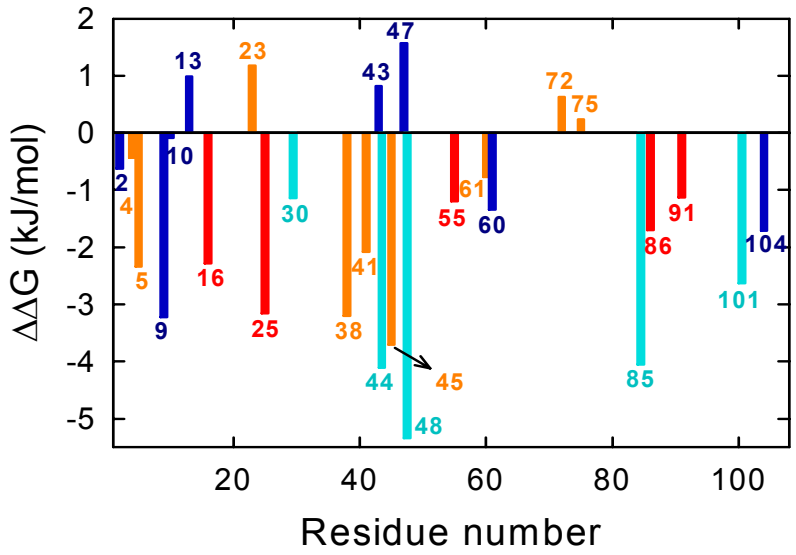


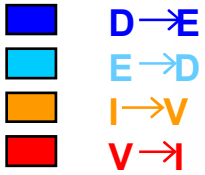


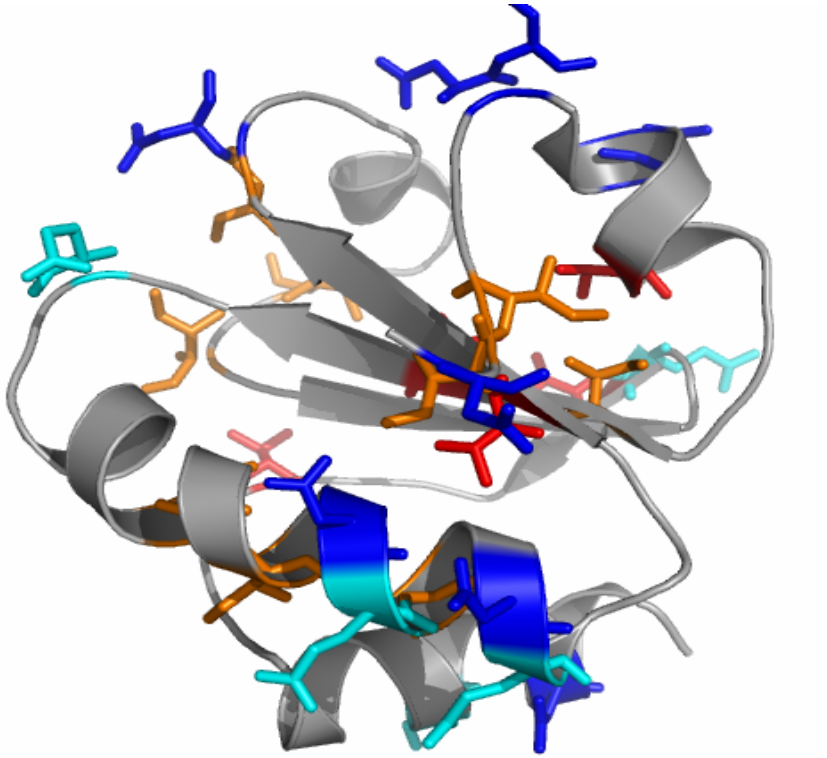
Rodriguez-Larrea, Minning, Borchert, Sanchez-Ruiz (2006) J. Mol. Biol., 360, 715-724.
Rodriguez-Larrea, Ibarra-Molero, Sanchez-Ruiz (2006) Biophys. J., L48-L50.

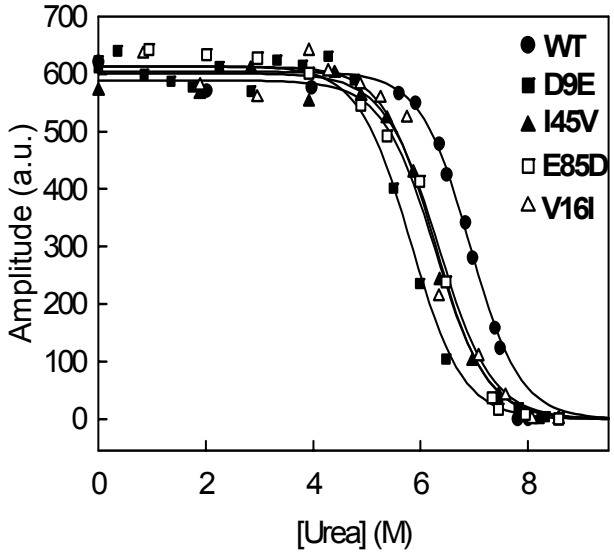
Kinetic stability is relevant in vivo

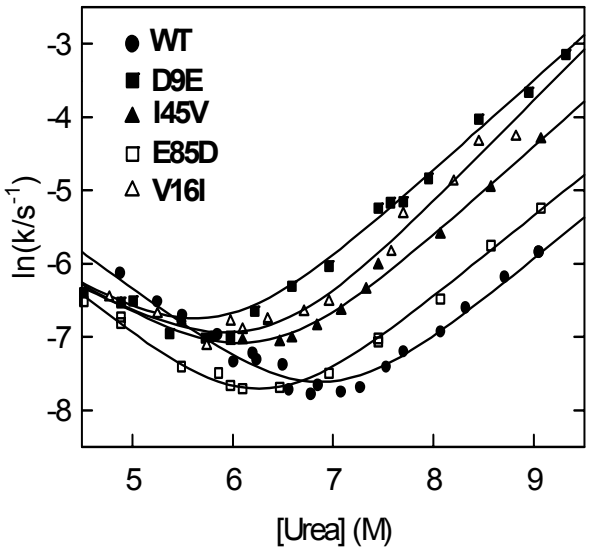
Natural selection for kinetic stability (a high free-energy barrier for unfolding) can be detected in sequence alignment analysis!

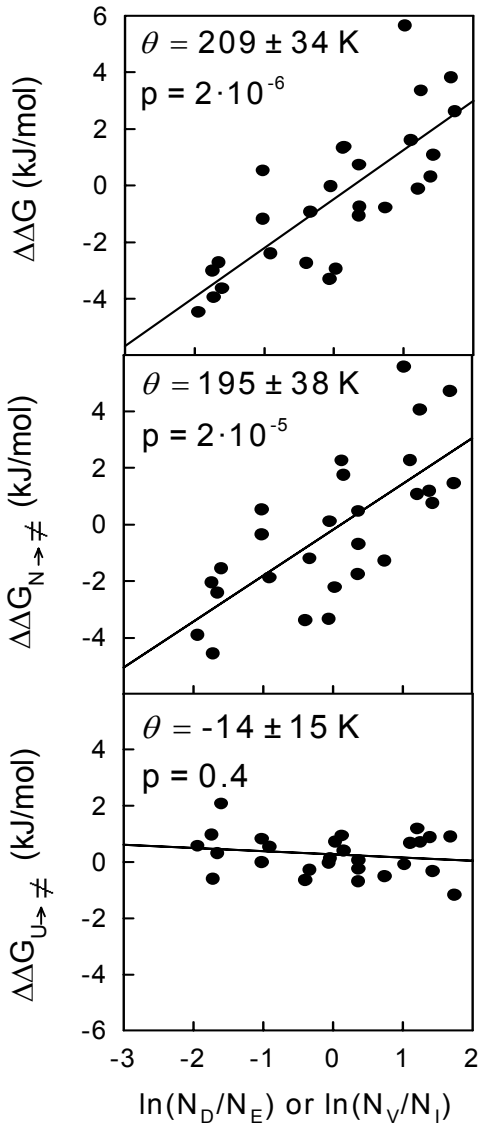










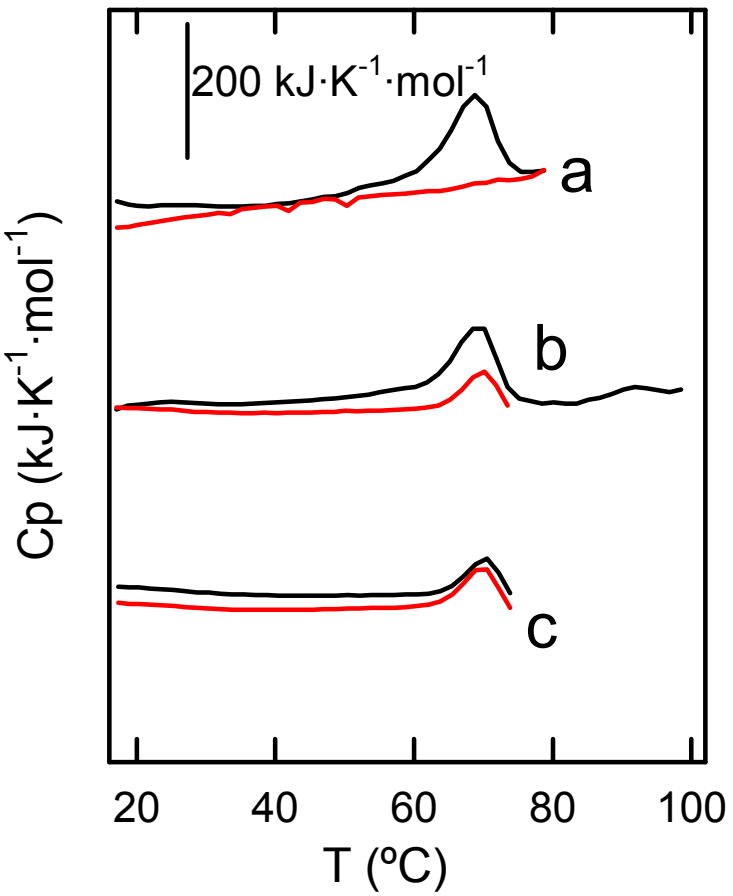


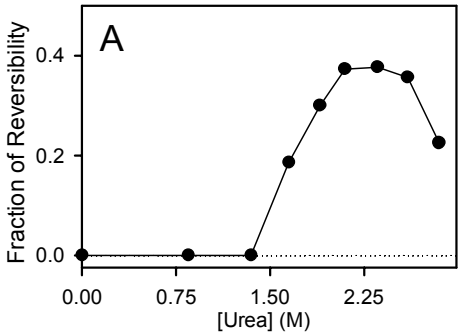
Unfolding kinetics

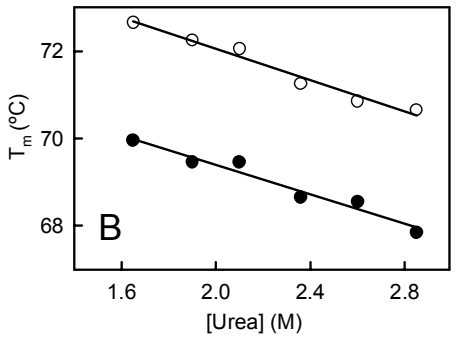
Godoy-Ruiz, Ariza, Rodriguez-Larrea, Perez-Jimenez, Ibarra-Molero, Sanchez-Ruiz (2006), J. Mol. Biol. 362, 966-978.

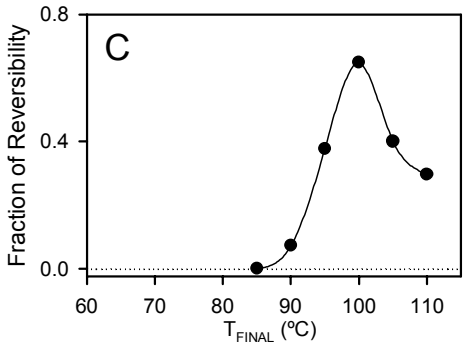
Kinetic stability may lead to alternative native or cuasi-native states

Thermal denaturation of lipase In urea solutions

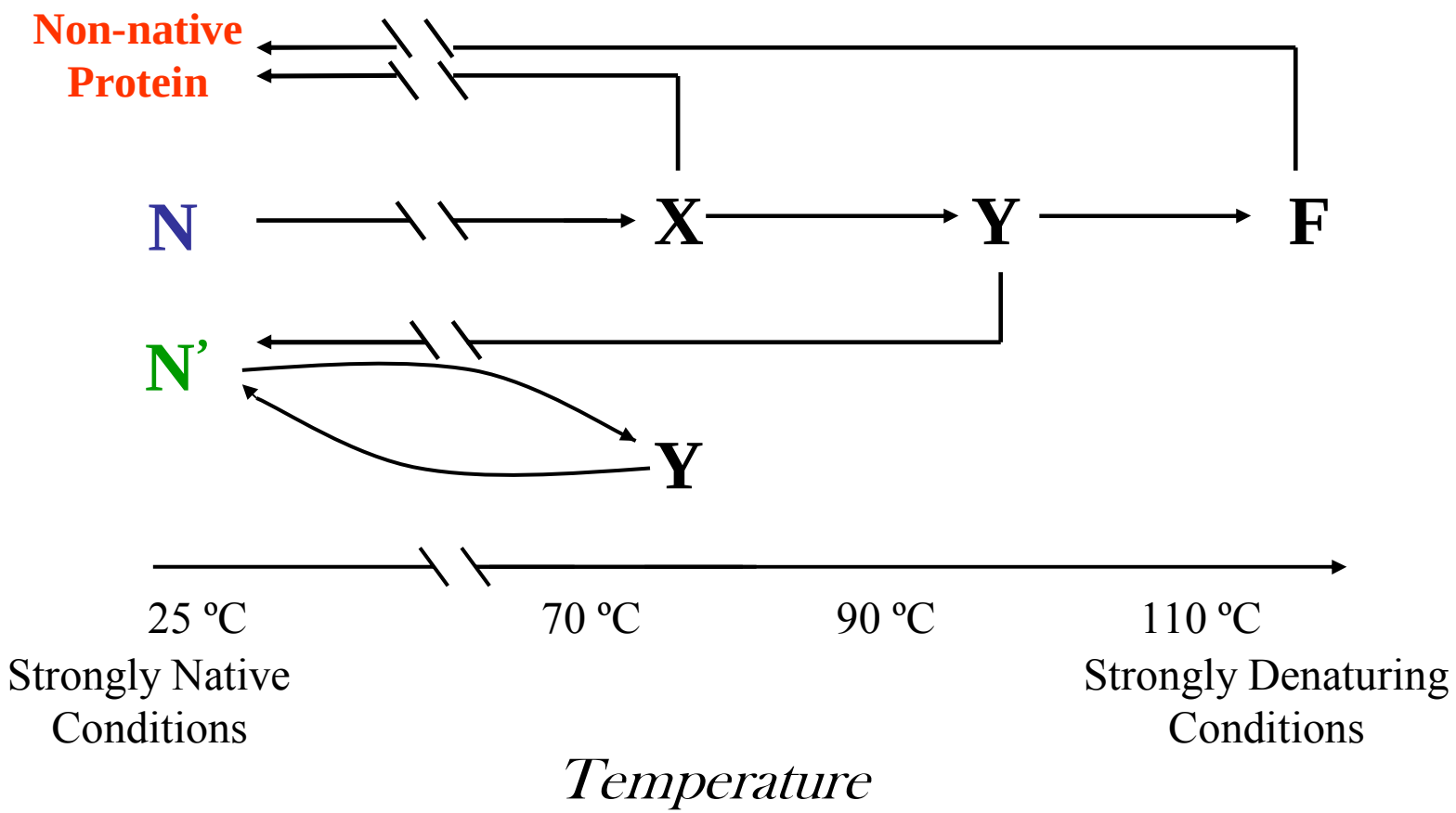








Rodriguez-Larrea, Ibarra-Molero, De Maria, Borchert, Sanchez-Ruiz (2007) Proteins, in press.



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THANKS!