

Protein Unfolding

DSC & DSF

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This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

DSC



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Calorimetry

Measurement of heat

Calorimeter

Instrument to measure heat in a process

Heat?

Energy different from work transferred in a process

First principle: $\Delta E = Q - W$

If T,P constant, then: $Q = Q_p = \Delta H$



DSC: Gold-standard for characterizing macromolecular stability

- Simple experimental set-up
- Widespread use in BioLabs
- Invaluable information on interactions (thermodynamic profile, conformational landscape, folding cooperativity)
- But... many words of caution concerning:
 - experimental set-up
 - data analysis
 - information accessible



► DSC provides invaluable information:

Stable conformation? YES/NO

K_{conf} , ΔG , T_m

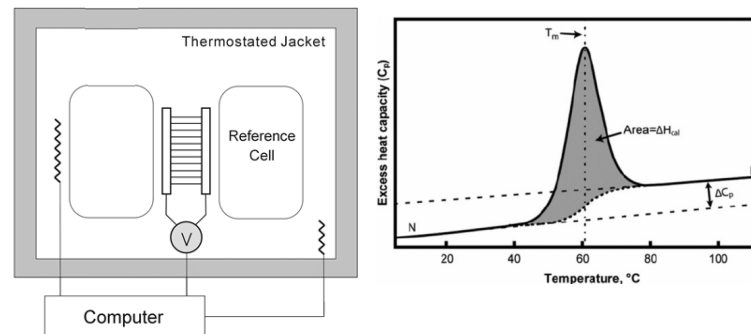
ΔH , $-T\Delta S$

ΔC_p , Δn_x

...

mutants

...



Very informative, but... very susceptible!



► DSC's “Black Legend”:

- Prone to artifacts (?)
- Difficult technique (data analysis) (?)
- Time consuming (?)
- Sample consuming (?)
- No kinetic information (?)



► DSC's “Golden Legend”:

- Low assay development
- In solution
- Non destructive (?)
- Universal signal
- Simple set-up
- Direct evidence of stability
- Global information on molecular stability
- Applicable to different systems (?)



► **DSC provides invaluable information:**

- **Thermodynamic and kinetic information on macromolecular stability (gold standard)**
- **Protein folding and conformational landscape**
- **Molecular basis of conformational disease**
- **Drug discovery and development**
 - Target engagement**
 - Lead selection and optimization**
- **Protein engineering and redesign**
- **Quality control and formulation**





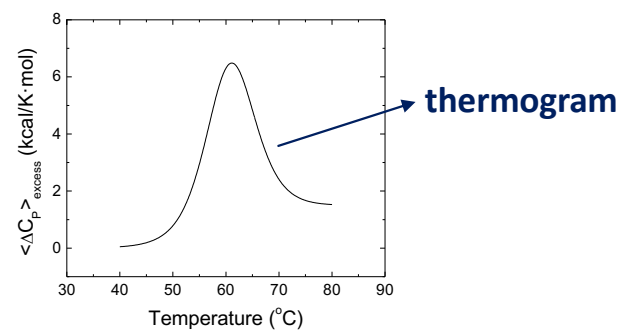
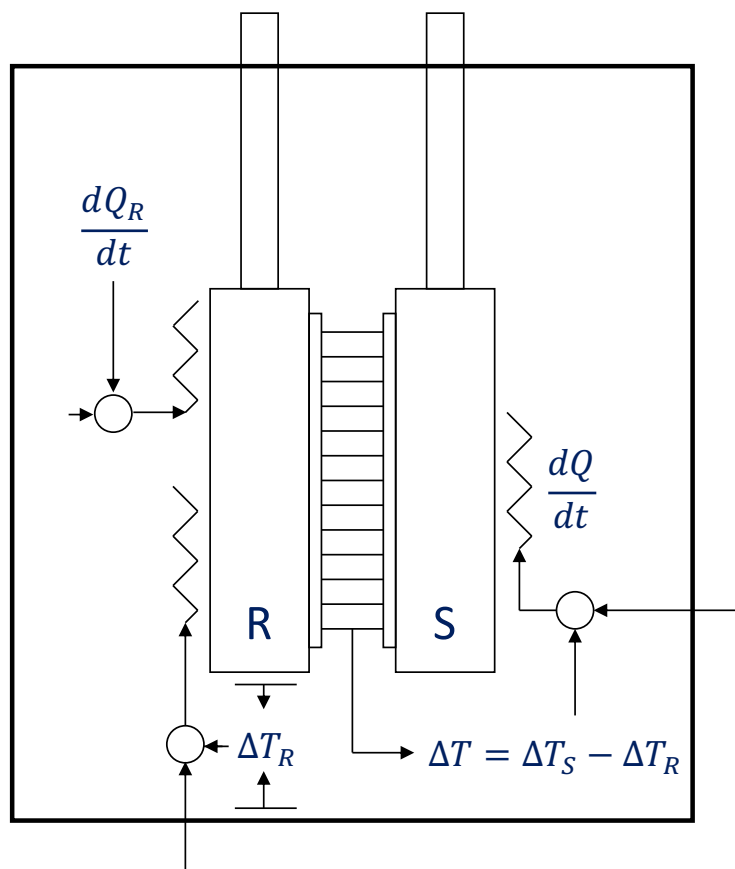
TA Instruments



MicroCal – Malvern-Panalytical



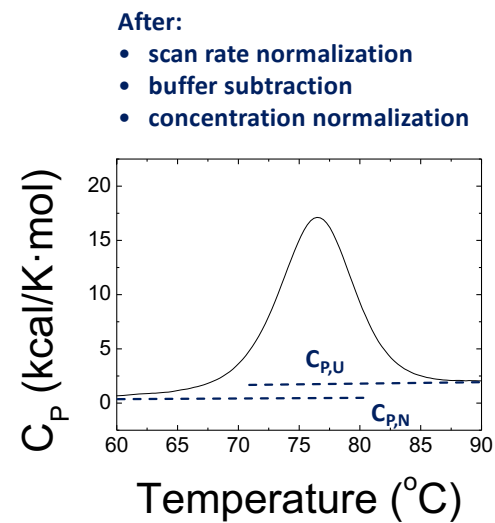
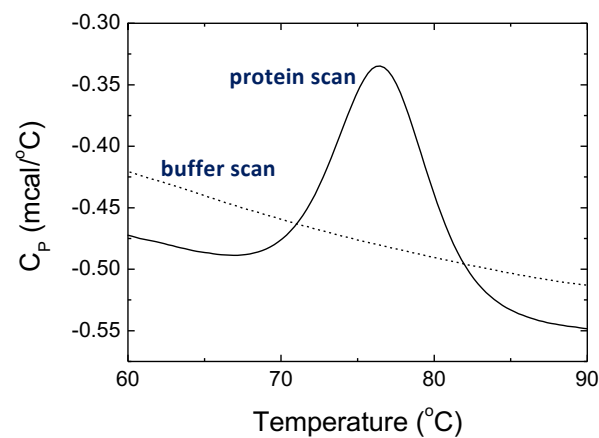
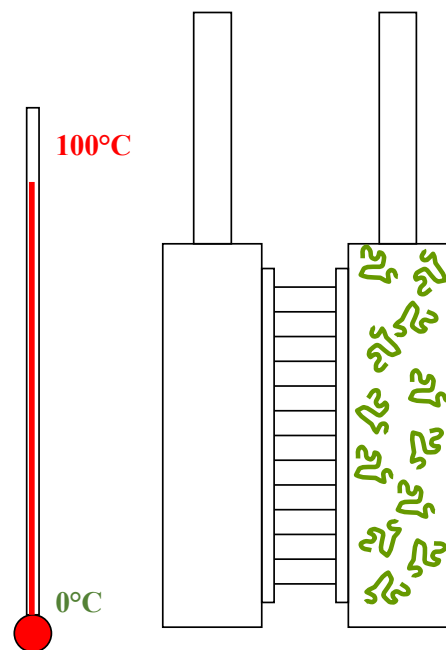
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Signal measured: Thermal power applied to sample cell to maintain ΔT close to zero, while increasing temperature

$$C_P = \frac{dQ}{dT} = \frac{dQ}{dt} \frac{1}{v} = \frac{dQ}{dt} \frac{1}{\frac{dT}{dt}}$$





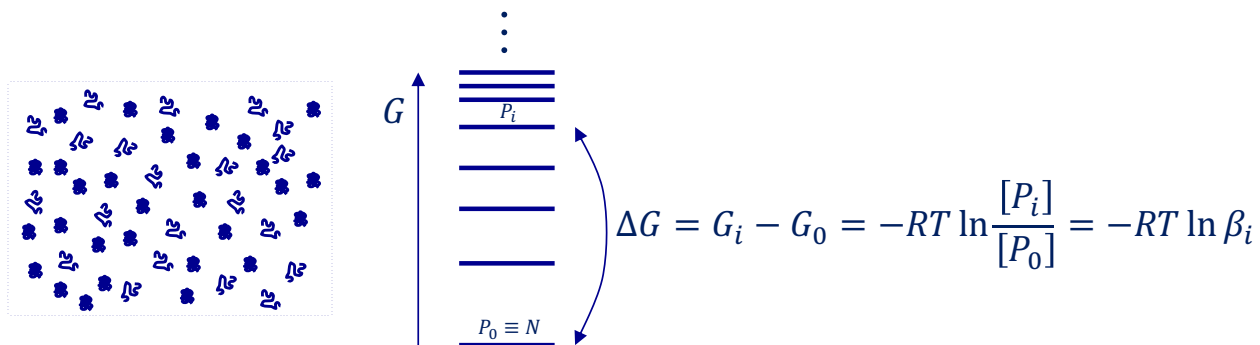
Measurement of the heat capacity of a macromolecule solution as a function of the temperature



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Stability

ΔG Gibbs energy of stabilization or unfolding
Difference in Gibbs energy between the native state
and the unfolded state



► Standard approach based on partition function Q

$$P_0 \leftrightarrow P_1 \leftrightarrow P_2 \leftrightarrow \dots \leftrightarrow P_i \leftrightarrow \dots \leftrightarrow P_n$$

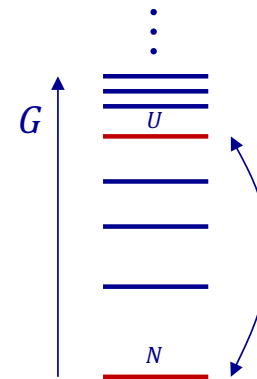
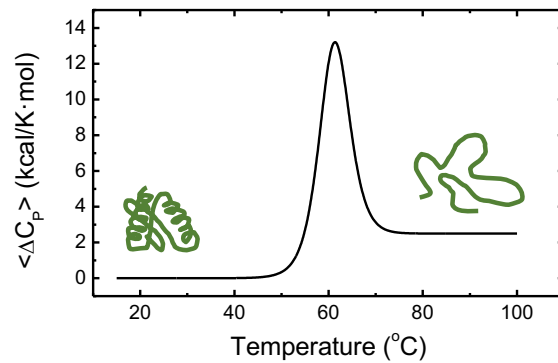
$$[P_i] = \beta_i [P_0]$$
$$[P_i] = \left(\prod_{r=1}^i K_r \right) [P_0]$$

$$Q = \sum_{i=0}^n \frac{[P_i]}{[P_0]} = \sum_{i=0}^n \beta_i$$

Wyman & Gill. "Binding and Linkage", University Science Books, 1990



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Cooperative unfolding → Reduction in the number of accessible states

Partially folded states are not significantly populated

Single-transition unfolding



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$$N \leftrightarrow U$$

i	G	ΔG	$\exp(-\Delta G/RT)$
fold	G_N	0	1
unfold	G_U	ΔG	K

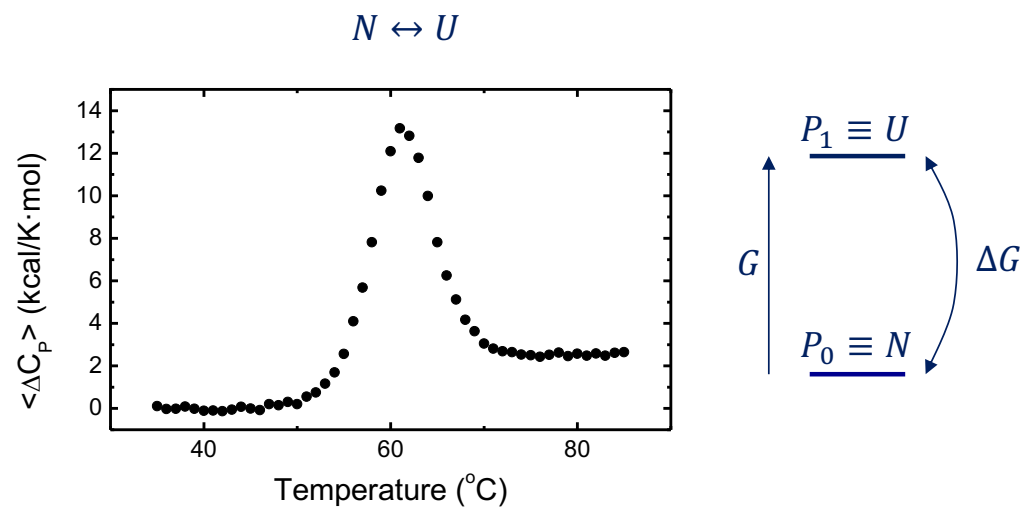
$$Q = 1 + \beta = 1 + K$$

$$\chi_N = \frac{1}{1 + K}$$

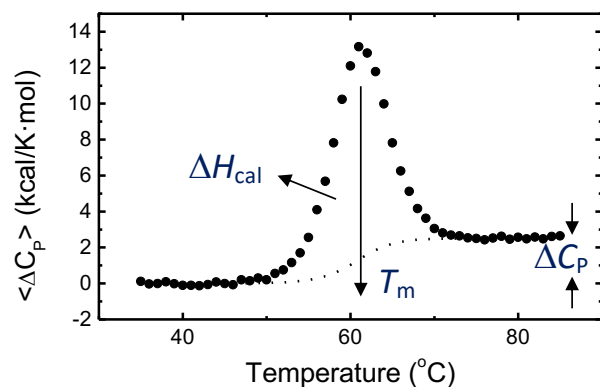
$$\chi_U = \frac{K}{1 + K}$$



► Single transition unfolding (two-state unfolding)

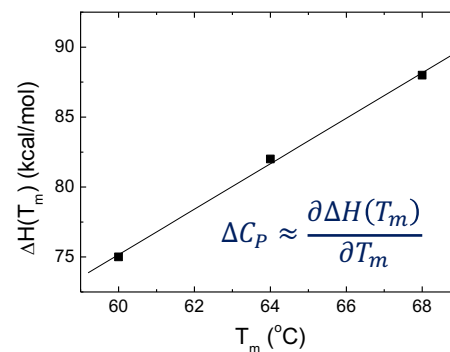


► Single transition unfolding: model-free analysis

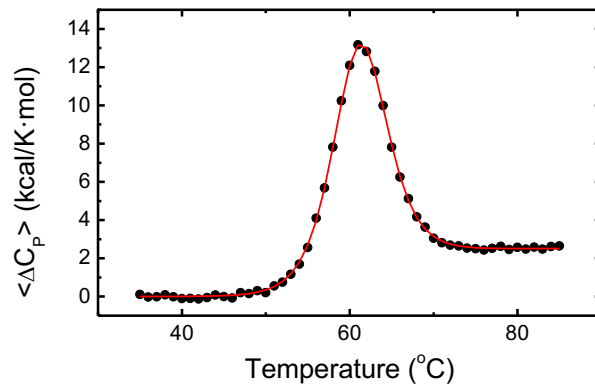


- T_m and ΔH : well determined
- ΔC_p : poorly determined

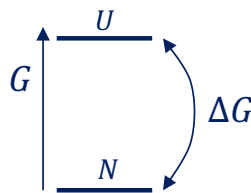
$$\Delta H(T_m) = \int_{T_0}^{T_1} \langle \Delta C_p \rangle dT$$
$$\Delta S(T_m) = \int_{T_0}^{T_1} \frac{\langle \Delta C_p \rangle}{T} dT$$



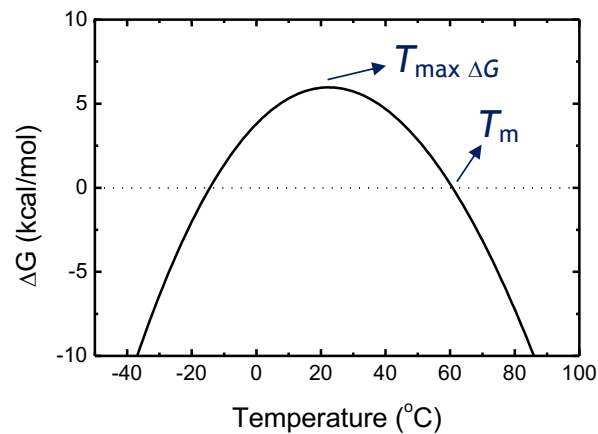
► Single transition unfolding: NLSF analysis

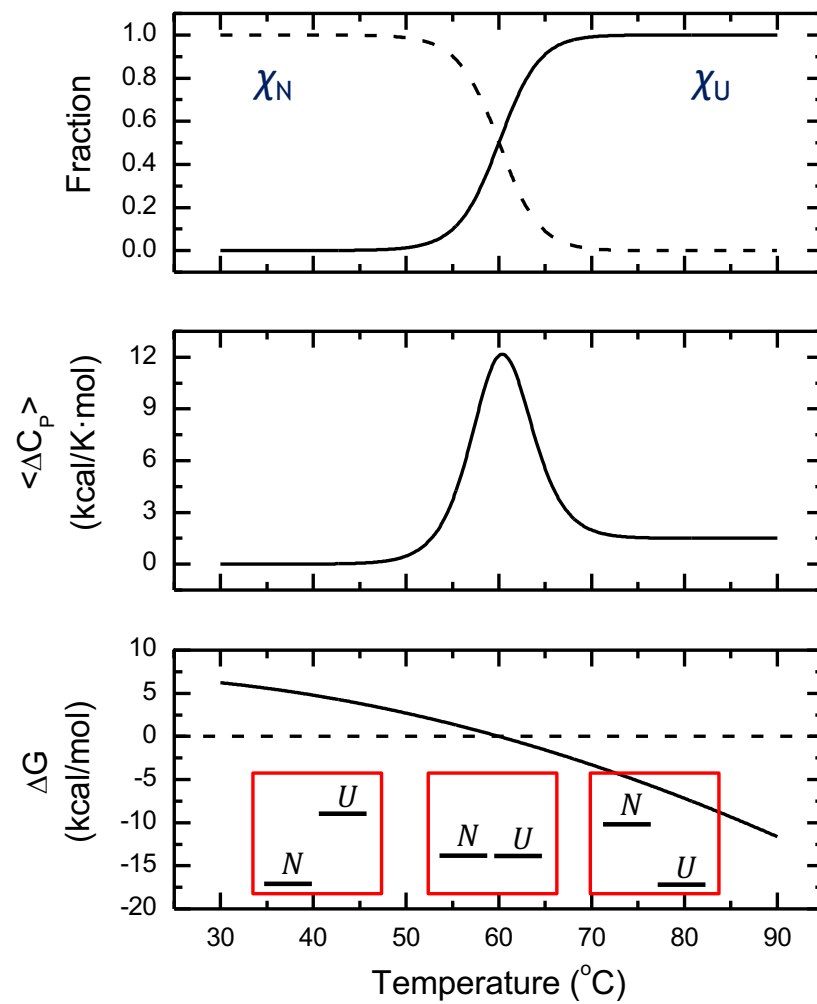


T_m 60.75 ± 0.03 °C
 $\Delta H(T_m)$ 102 ± 0.3 kcal mol⁻¹
 ΔC_p 2.51 ± 0.02 kcal K⁻¹ mol⁻¹



Stability profile $\Delta G(T)$



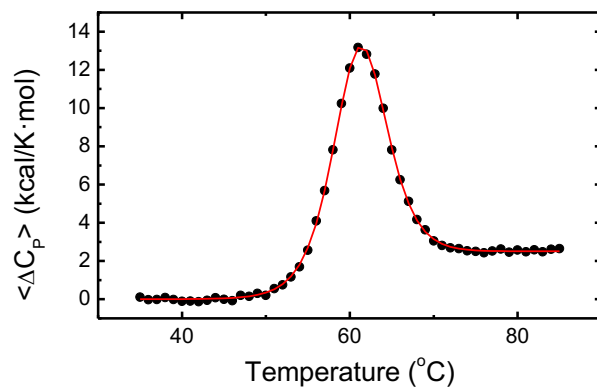


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For a typical globular protein:

T_m	$\sim 50-70\text{ }^{\circ}\text{C}$
$\Delta H(T_m)$	$\sim 80-120\text{ kcal mol}^{-1}$
ΔC_p	$\sim 2-3\text{ kcal K}^{-1}\text{ mol}^{-1}$
$\Delta G(25\text{ }^{\circ}\text{C})$	$\sim 5-10\text{ kcal mol}^{-1}$
$T_{\max\Delta G}$	$\sim 20-30\text{ }^{\circ}\text{C}$
T_f	$< 0\text{ }^{\circ}\text{C}$

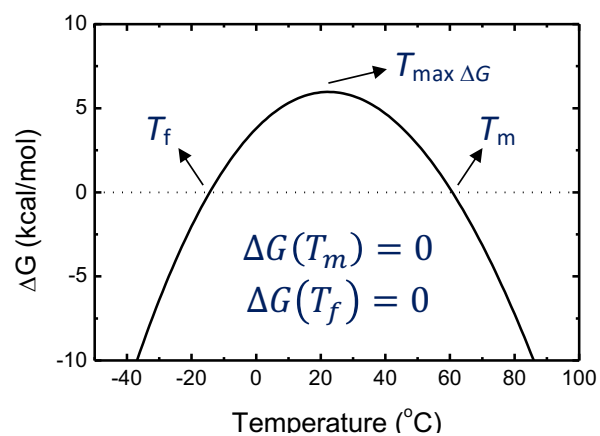




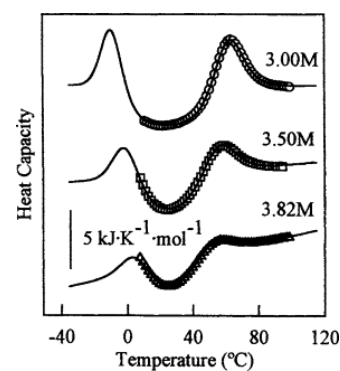
$$T_m = 60.75 \pm 0.03 \text{ } ^\circ\text{C}$$

$$\Delta H(T_m) = 102 \pm 0.3 \text{ kcal/mol}$$

$$\Delta C_p = 2.51 \pm 0.02 \text{ kcal/K}\cdot\text{mol}$$

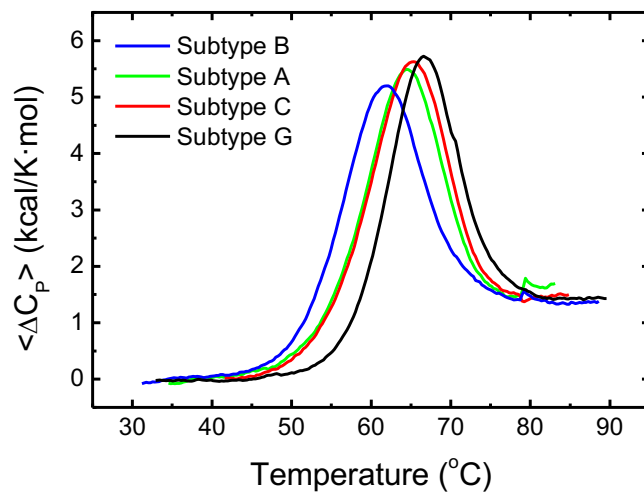


Possibility of cold denaturation!
(under certain conditions/circumstances)



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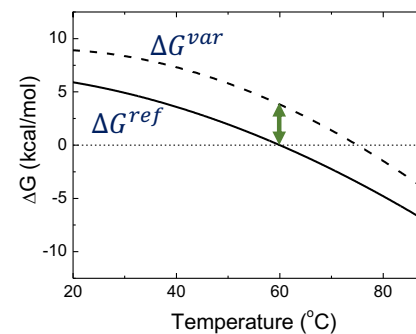
► Can we observe stability differences?



Problem: $\Delta G = \Delta G(T)$



Employ a common temperature!



$$\Delta\Delta G = \Delta G^{var}(T_m^{ref}) - \Delta G^{ref}(T_m^{ref}) = \Delta G^{var}(T_m^{ref})$$





$$\Delta\Delta G \approx \frac{\Delta H^{var}(T_m^{var})}{T_m^{var}} (T_m^{var} - T_m^{ref}) \approx \Delta S^{ref}(T_m^{ref}) \Delta T_m$$



Two-transition unfolding



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i	ΔG_i	$\exp(-\Delta G_i/RT)$
	0	1
	ΔG_1	K_1
	ΔG_2	K_2
	$\Delta G_1 + \Delta G_2$	$K_1 K_2$

$$Q = 1 + \beta_1 + \beta_2 + \beta_3 = 1 + K_1 + K_2 + K_1 K_2$$

$$\chi_N = \frac{1}{1 + K_1 + K_2 + K_1 K_2} \quad \chi_{I_2} = \frac{K_2}{1 + K_1 + K_2 + K_1 K_2}$$

$$\chi_{I_1} = \frac{K_1}{1 + K_1 + K_2 + K_1 K_2} \quad \chi_U = \frac{K_1 K_2}{1 + K_1 + K_2 + K_1 K_2}$$



i	ΔG_i	$\exp(-\Delta G_i/RT)$
	0	1
	ΔG_1	K_1
	$\Delta G_1 + \Delta G_2$	$K_1 K_2$

$$Q = 1 + \beta_1 + \beta_2 = 1 + K_1 + K_1 K_2$$

$$\chi_N = \frac{1}{1 + K_1 + K_1 K_2}$$

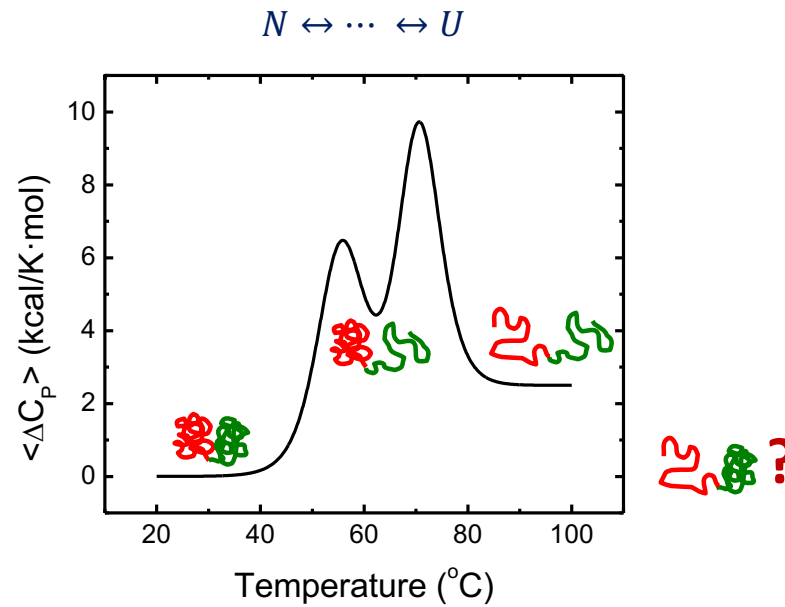
$$\chi_{I_2} \approx 0$$

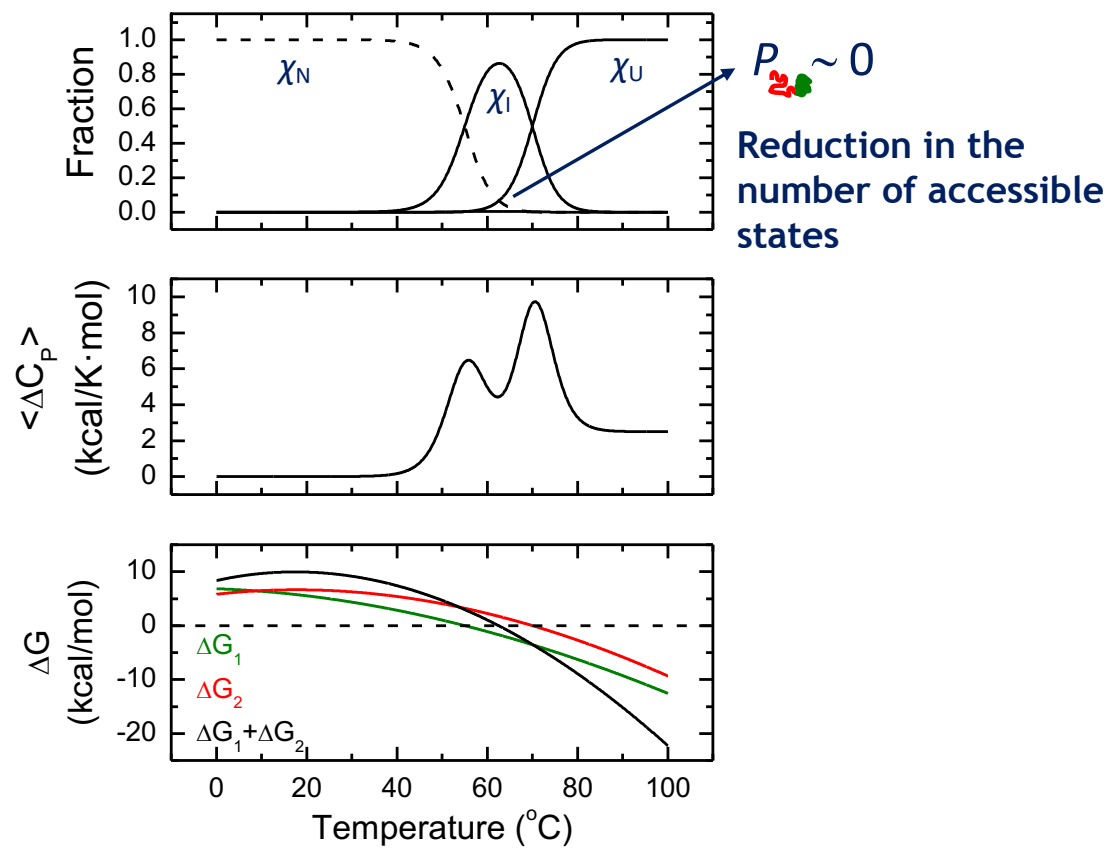
$$\chi_{I_1} = \frac{K_1}{1 + K_1 + K_1 K_2}$$

$$\chi_U = \frac{K_1 K_2}{1 + K_1 + K_1 K_2}$$

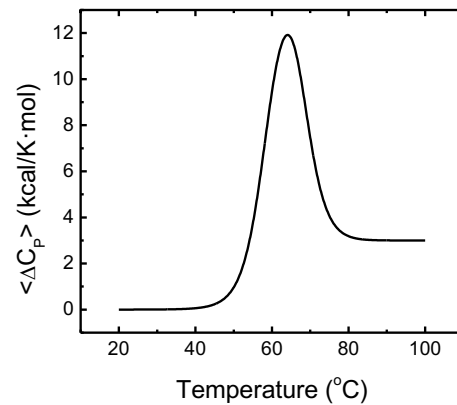


► Two-transition unfolding (three-state unfolding?)





► How many transitions?



Two-state test (single transition test):

- Overlapping of unfolding curves obtained with different techniques (spectroscopy)
- van't Hoff-calorimetric enthalpies ratio (calorimetry) $\frac{\Delta H_{vH}}{\Delta H_{cal}}$

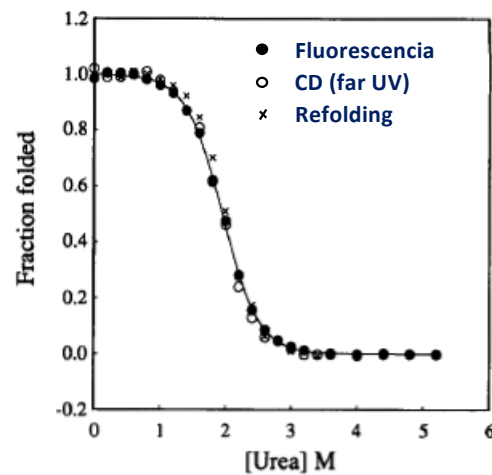


Be careful...

- **Overlapping unfolding curves from different techniques is a necessary but not sufficient condition for a single transition**
- **Spectroscopy may report global and local unfolding information**
- **A single transition implies total cooperativity (absence of intermediate partially unfolded states)**
- **Uncertainties and noise may mask details and phenomena**

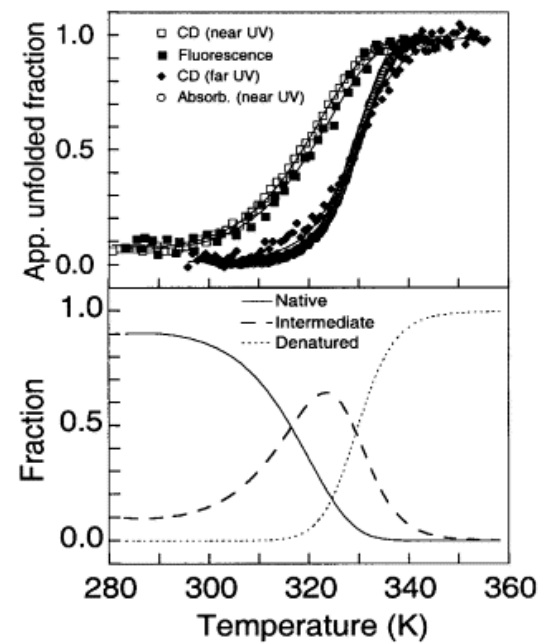


Single transition



Genzor et al. *Protein Science* 1996 **5** 1376-1388

Two transitions



Irún et al. *Journal of Molecular Biology* 2001 **306** 877-888



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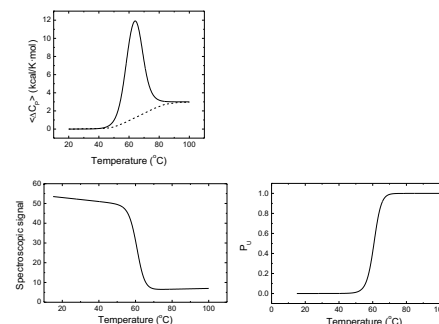
van't Hoff enthalpy

$$\Delta H_{vH} = RT^2 \frac{\partial \ln K}{\partial T}$$

$$\Delta H_{vH} = \frac{4RT_m^2 C_{P,max}}{\Delta H_{cal}}$$

$$\Delta H_{vH} = 4RT_m^2 \left(\frac{\partial \chi_U}{\partial T} \right)_{T_m}$$

model-free analysis



$$\frac{\Delta H_{vH}}{\Delta H_{cal}} = 1 \quad \text{cooperative unit} \equiv \text{molecule (single transition)}$$

$$N \leftrightarrow U$$

$$\frac{\Delta H_{vH}}{\Delta H_{cal}} < 1 \quad \text{cooperative unit} < \text{molecule (more than one transition)}$$

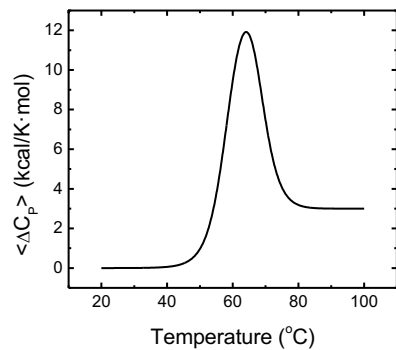
$$N \leftrightarrow I \leftrightarrow \dots \leftrightarrow U \quad \frac{\Delta H_{vH}}{\Delta H_{cal}} \neq \frac{1}{n_D}$$

$$\frac{\Delta H_{vH}}{\Delta H_{cal}} > 1 \quad \text{cooperative unit} > \text{molecule (self-associated native state)}$$

$$N_n \leftrightarrow nU \quad \frac{\Delta H_{vH}}{\Delta H_{cal}} \neq n \quad \frac{\Delta H_{vH}}{\Delta H_{cal}} = 4 \left(\frac{n - \sqrt{n}}{n - 1} \right)^2$$

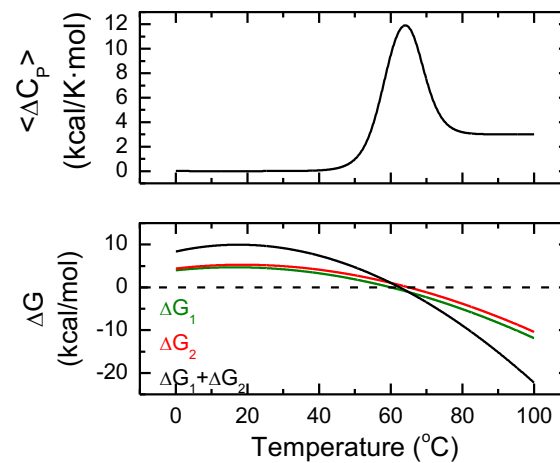
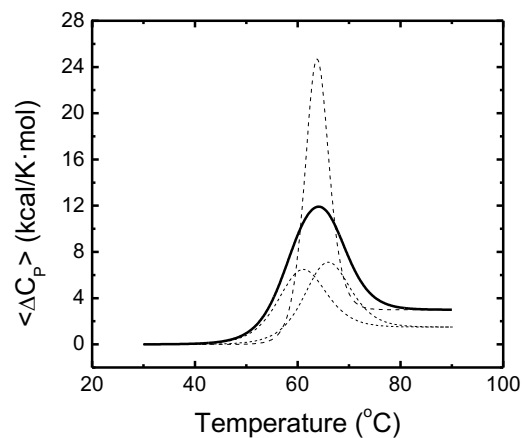
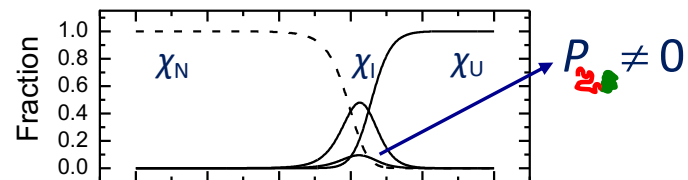


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ΔH_{cal} 144.3 kcal mol⁻¹
 T_m 63.5 °C
 $C_{p\text{max}}$ 10.2 kcal K⁻¹ mol⁻¹

ΔH_{vH} 63.4 kcal mol⁻¹
 $\Delta H_{\text{vH}}/\Delta H_{\text{cal}}$ 0.44






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Unfolding-Binding Coupling

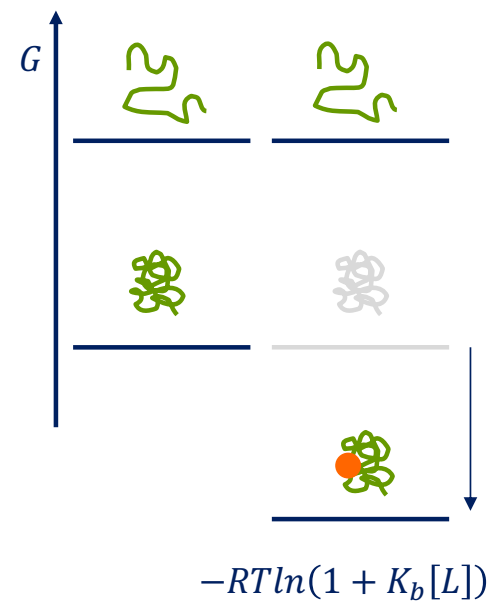
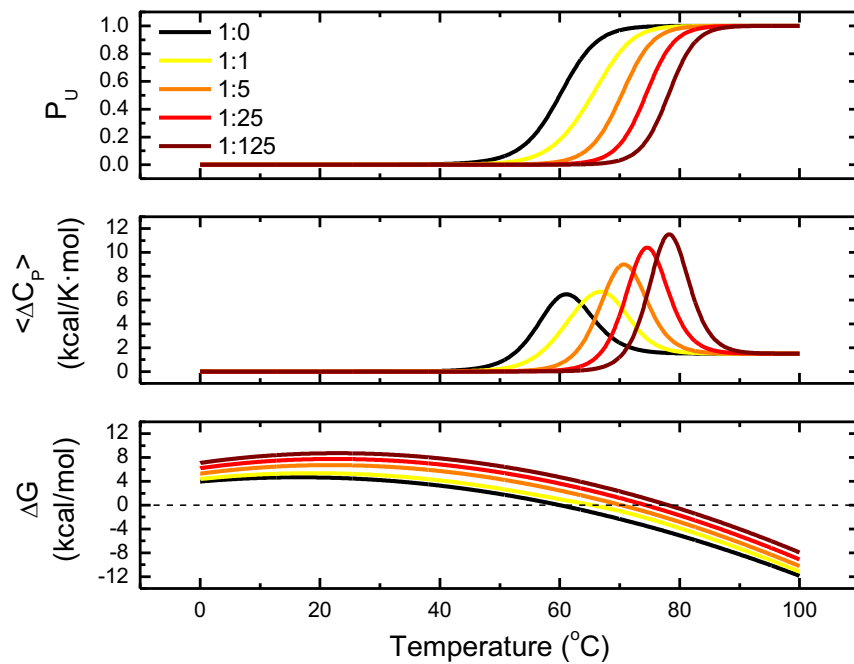


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i	ΔG_i	$\exp(-\Delta G_i/RT)$
	0	1
	ΔG_{bind}	$K_a[L]$
	ΔG^0	K^0

$$Q = 1 + K_a[L] + K^0$$








- Indirect evidence for interaction
- Determination of binding affinity (?)

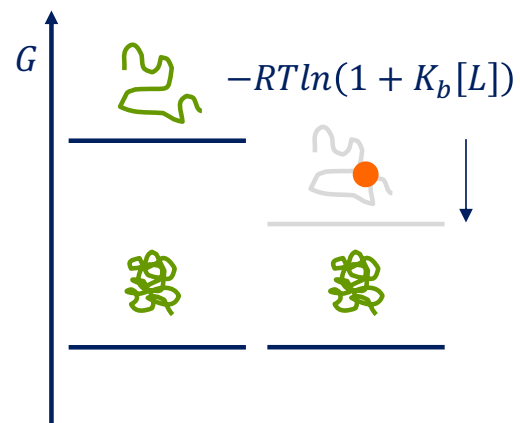
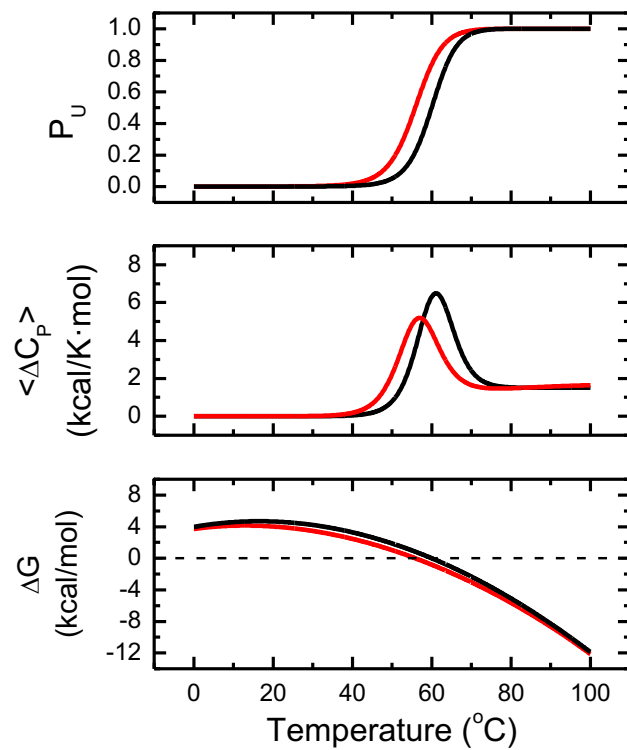


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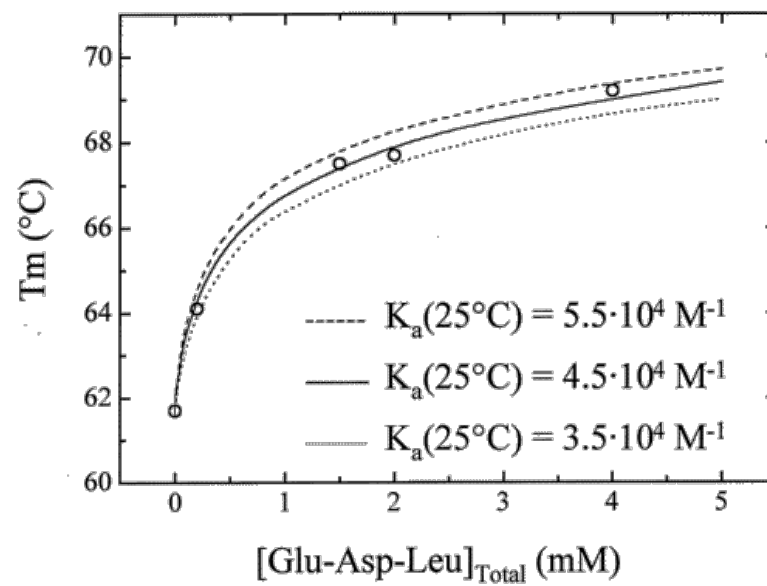
i	ΔG_i	$\exp(-\Delta G_i/RT)$
	0	1
	ΔG^0	K^0
	$\Delta G^0 + \Delta G_{\text{bind}}$	$K^0 K_a[L]$

$$Q = 1 + K^0(1 + K_a[L])$$





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DSF



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DSF Monitoring protein unfolding using an extrinsic fluorescent reporter (e.g., ANS, SYPRO Orange)

Motivation:

- **Low throughput of conventional techniques (sample amount, number of assays)**
- **Conventional fluorescence plate readers do not allow broad temperature ramp**
- **Real-time qPCR allow broad temperature ramp, but do not record in the tryptophane fluorescence range**



DSF

Thermofluor® or Thermal Shift Assay (TSA)

Stabilization effect induced by ligand binding or by solvent components on the thermal stability of a protein

“Differential”

Usually, differences between a “reference” sample and “test” samples are measured



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FluoDia T70 (PTI)



Prometheus (Nanotemper)



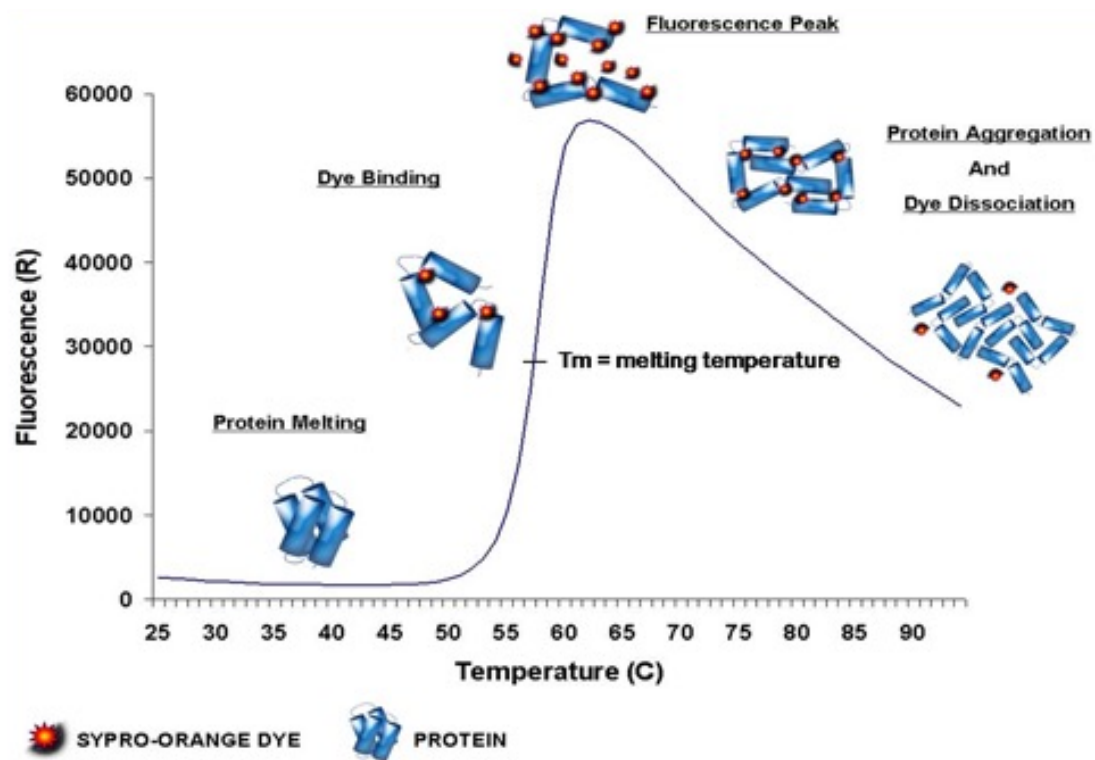
CFX Opus qPCR (BioRad)



AriaMx qPCR (Agilent)



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By Argonne National Laboratory -
http://www.bio.anl.gov/molecular_and_systems_biology/Sensor/sensor_images/



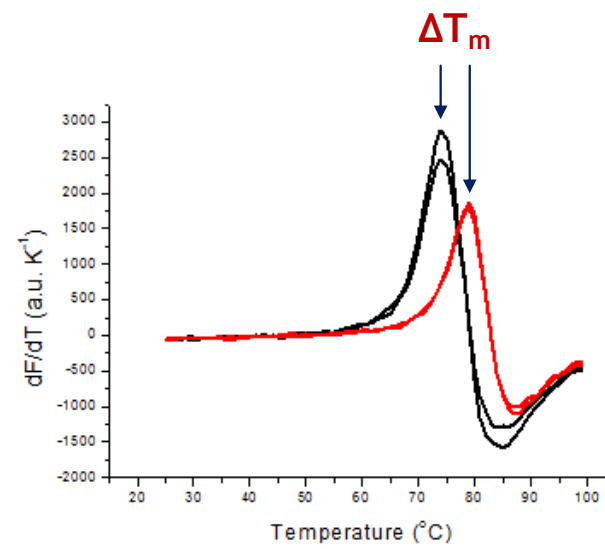
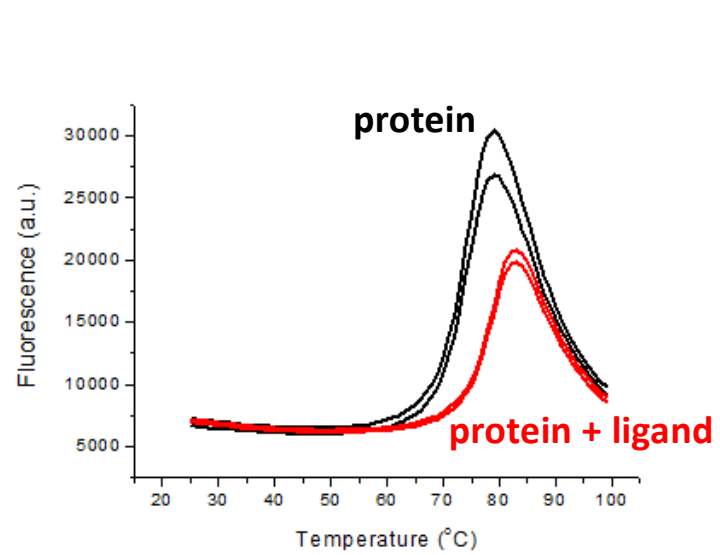
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DSF **Now, also using intrinsic tryptophan or cofactors fluorescence**

Applications:

- Drug screening
- Drug lead optimization (**Extent of stabilization does not correlate with binding affinity!**)
- Studies of enzyme mechanism
- Protein stabilization for optimized isolation
- Characterization of engineered proteins
- Optimization of protein crystallization conditions
- Screening for inhibitors of protein-protein interactions of modulators of protein conformational changes
- Membrane proteins
- Decrypting proteins of unknown biological function
- CETSA, for cellular thermal shift assay





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