Calorimetry of biological macromolecules:

Theory and applications of the Nano-DSC

TA Instruments

2019

Schedule

1. Overview of NanoDSC Instrument and Theory
2. Applications
3. Sample Preparation, Data Analysis and Maintenance
4. Case Studies
5. Tour

TA Instruments, Inc.

- Design, Manufacture and Service
  - Microcalorimeters
  - Thermal analysis Instrumentation
  - Dynamic Vapor Sorption Instrumentation
  - Rheometers and Dynamic Mechanical Analyzers
- Subsidiary of Waters Corp since 1995
- ~500 employees
- Direct sales, service and support in: China, Taiwan, Japan, Korea, India, UK, France, Germany, Belgium, Netherlands, Sweden, Italy, Spain, Australia, Mexico, Brazil
- Manufacturing facilities in Delaware, Germany, and **Utah**.

TA Instruments Microcalorimetry Product Line

NanoDSC Autosampler

MC DSC

TAM IV

SolCal

NanoITC
Std Vol & Low Vol

AffinityITC Autosampler

TAM Air

Why use a Nano DSC?

- DSC is the only technique that allows the direct measure of T_m, ΔC_p and ΔH.
  - DSC allows for calculation of entropy (ΔS) and free energy (ΔG).
- Does not require nitrogen purge
  - i.e. CD-spectroscopy
- Equally useful for macromolecules and small molecules (no MW limit)
- Compatible with essentially any buffer or additive
- Requires small sample concentrations and volume

Nano DSC Specifications and Configurations

Temp Range	**-10° C to 130° C Capillary** **-10° C to 160° C Cylindrical**
Pressure Perturbation	**To 6 atm**
Cell volume	**0.3 mL**
Cell material	**Platinum – Capillary** **Gold – Cylindrical**

Capillary can also be automated

Nano DSC Cell Geometry

Continuous Capillary (130˚)

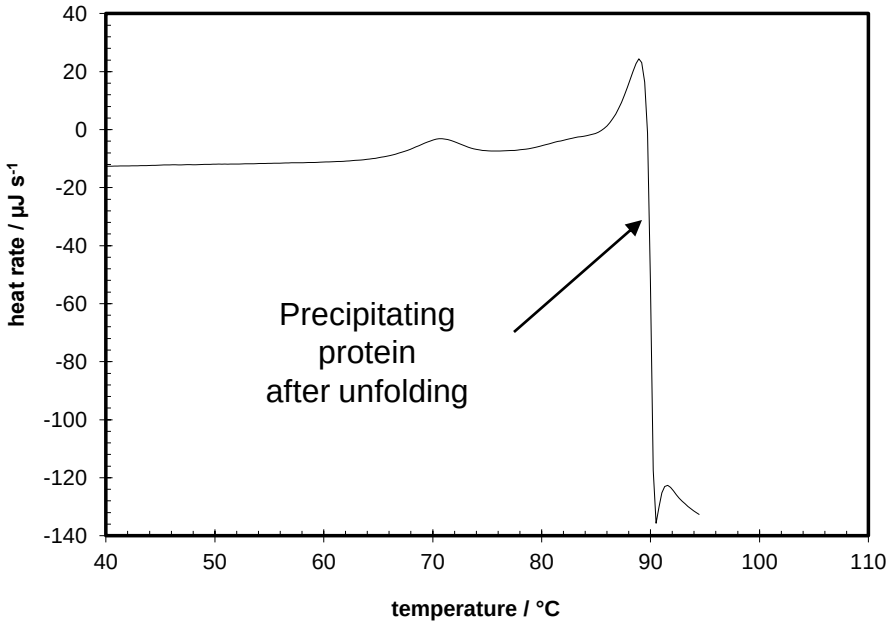
Fixed-cell for maximum sensitivity

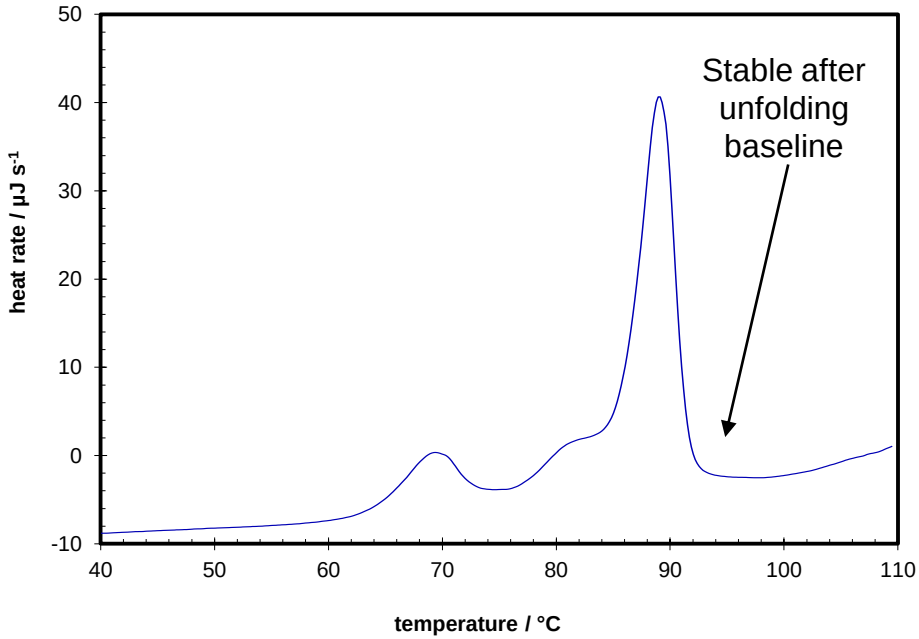
- Cell Construction; Inert to biomaterials 99.99% Platinum
- Small Sample Volume (0.3 mL)
- Attenuates or delays onset of aggregation until after protein has unfolded
- Easy-to-fill and clean design

Benefit of Capillary Sample Cell

Data obtained with a DSC with “Coin and Cylindrical ” shaped sample cell

Data obtained with a Nano DSC with continuous Capillary sample cell





Purified human IgG_1 monoclonal antibody in physiological buffer; 0.5 mg/mL

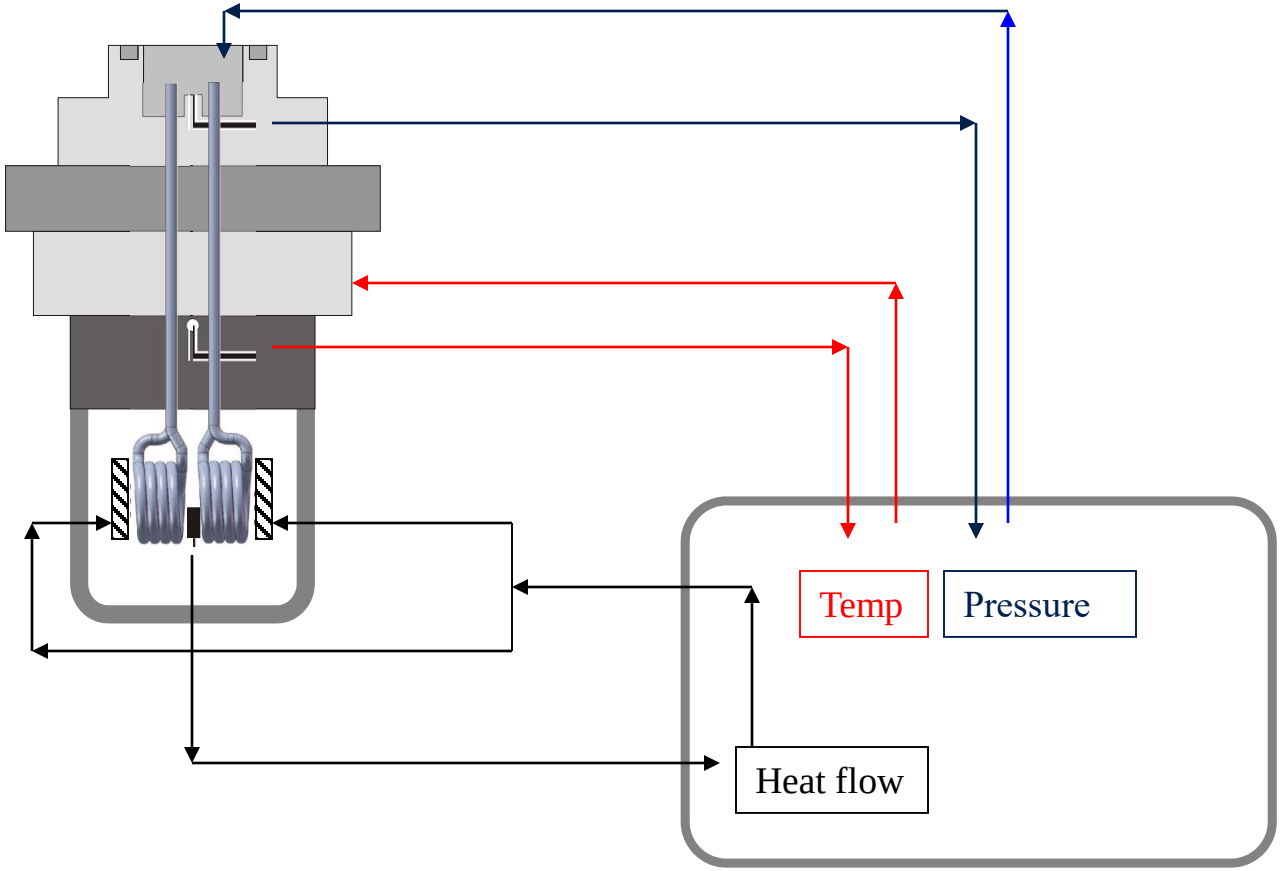
Nano DSC Optional Cell Geometry

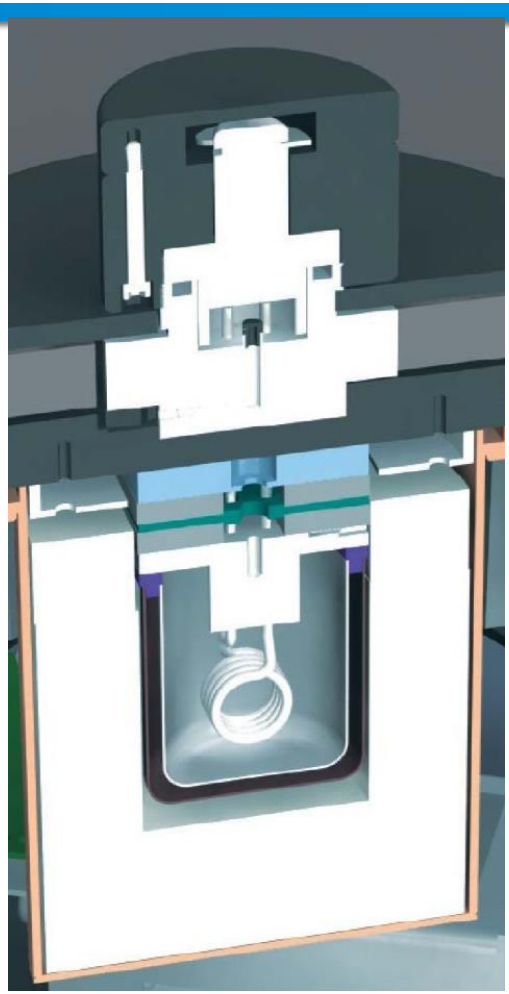
Cylindrical

High-Temperature Version (160°C)

- Cell Construction; 99.999% **Gold**
- Small Sample Volume (0.33 mL)
- High temperature option
- Easy-to-fill and clean

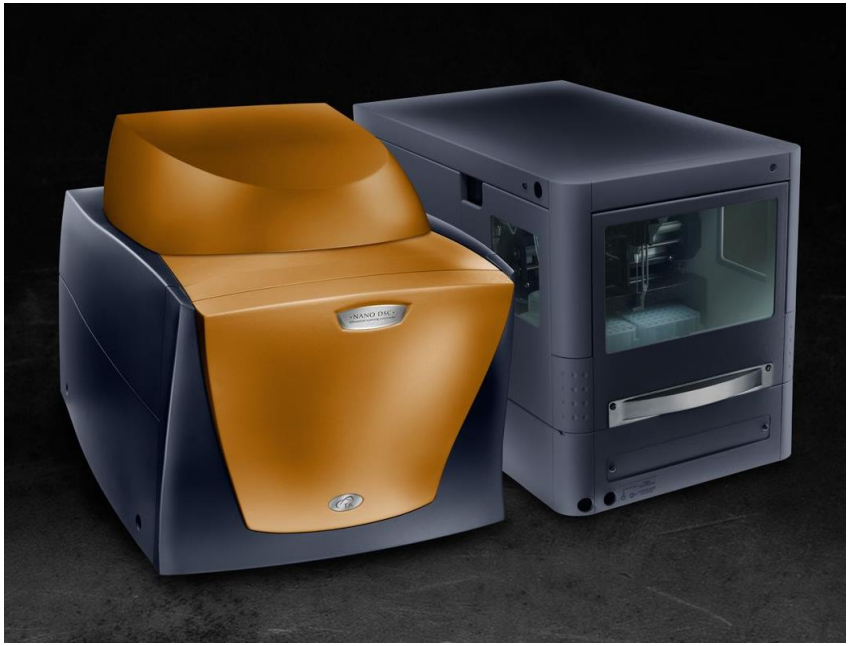
Nano DSC Schematic





Software – Firmware control

Nano DSC Automation System

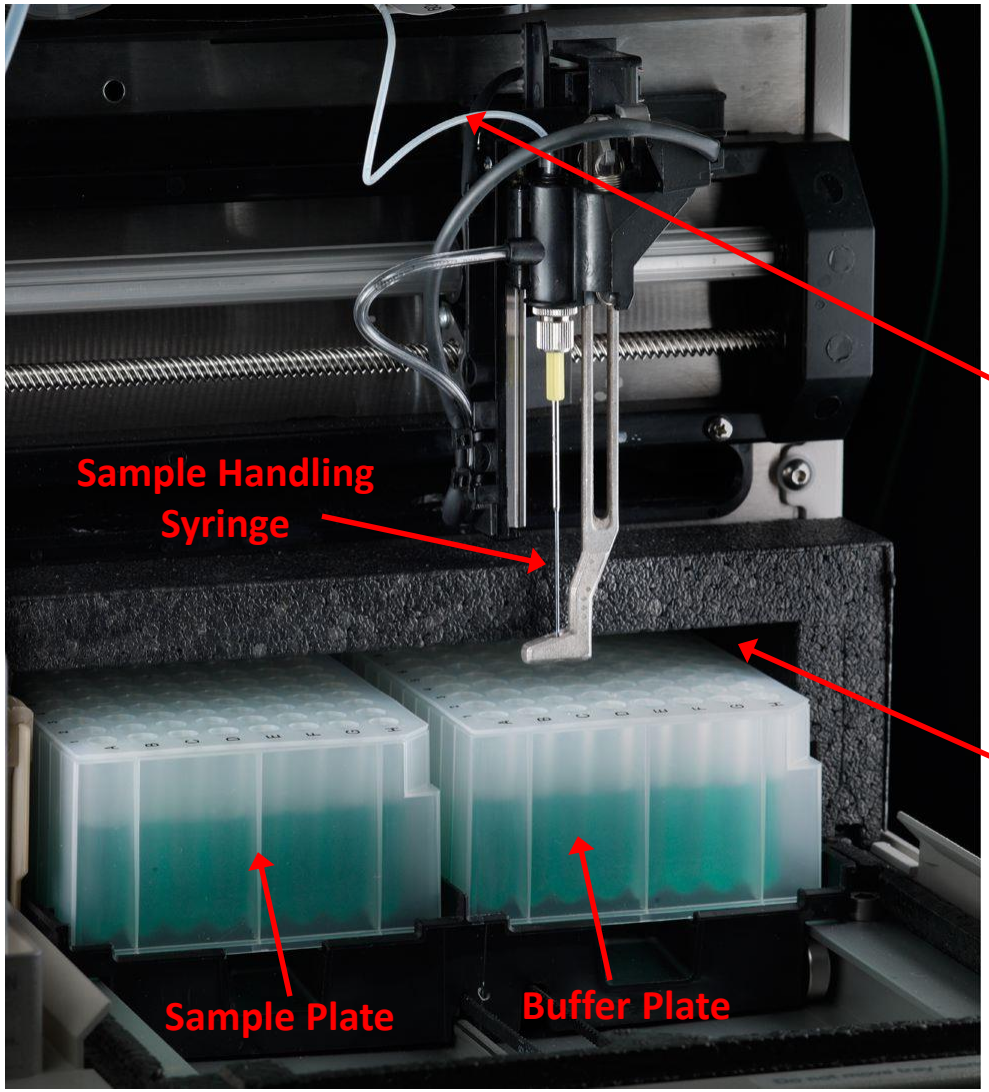


- Both Nano DSC and Autosampler programmed from same software

Nano DSC Autosampler System:

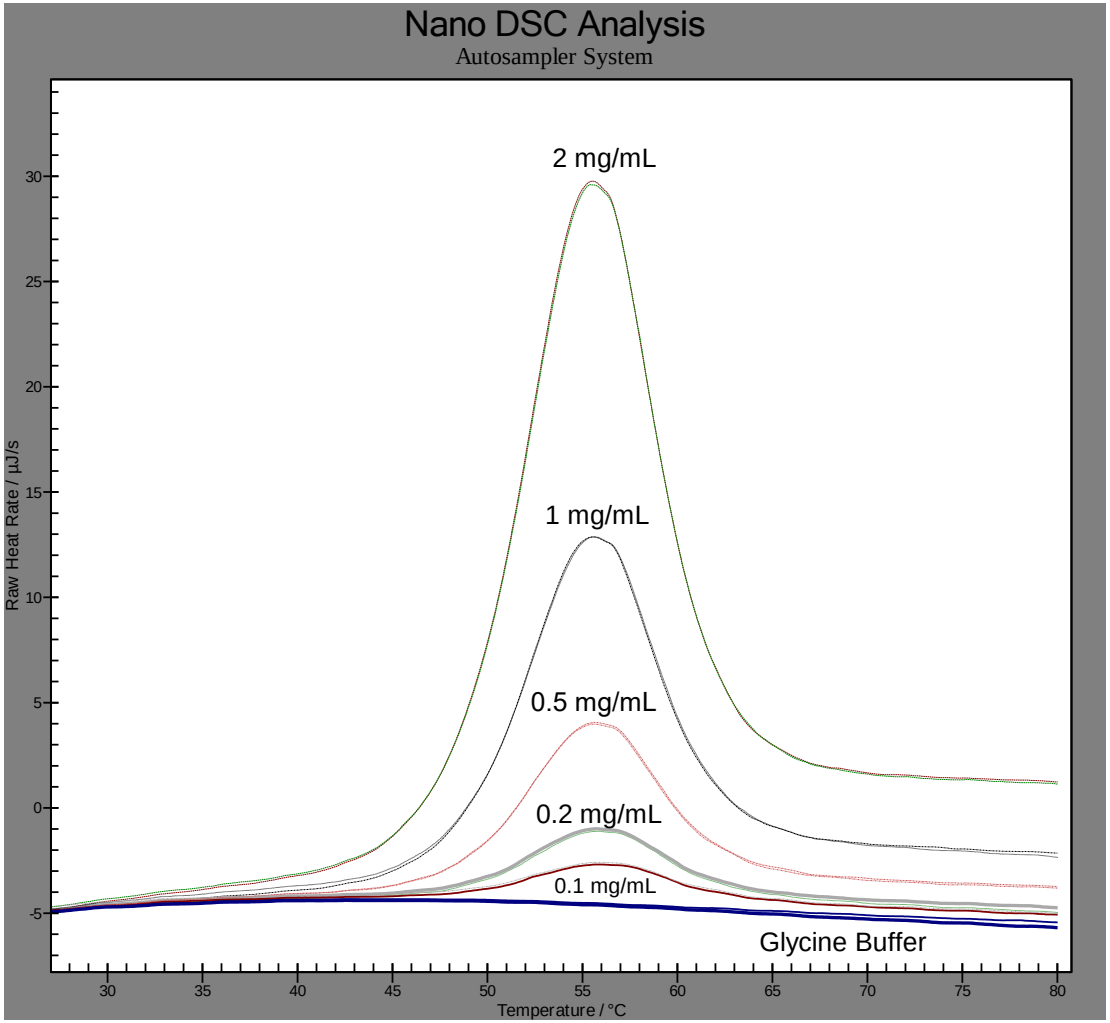
- Program-and-walk-away functionality
- Liquid handling autosampler from Spark Holland
- Temperature controlled sample storage (4°C – Ambient)
- Two 96-well sample plates
- Programming for up to 96 samples with matching buffers

Nano DSC Autosampler



Nano DSC Autosampler Performance

Raw Heat Rate



Experiment Parameters:

Instrument: Nano DSC
Sample: Lysozyme
Scan Range: 10 – 80ºC
Scan Rate: 1ºC / min
Sample Buffer: Glycine
Sample Vol: 1mL

A/S & DSC Protocol:

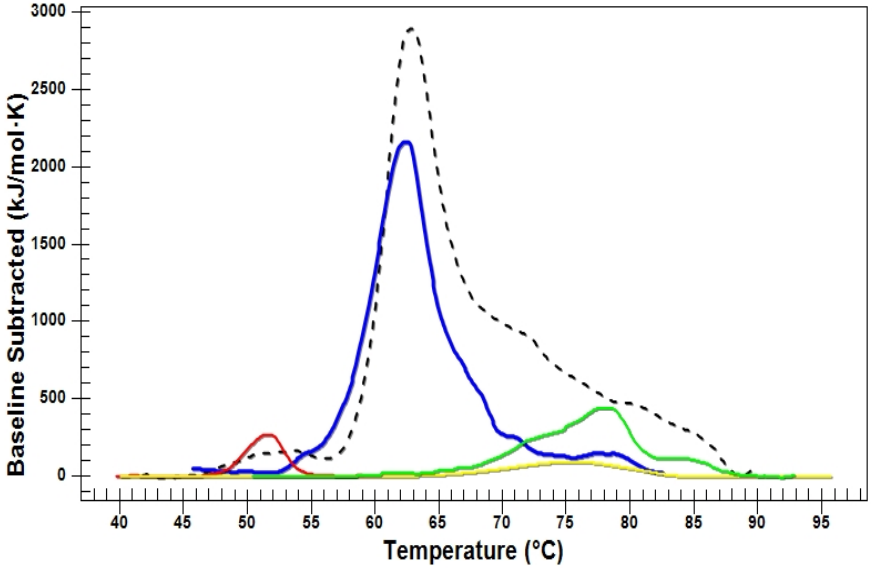
1. Initial cell wash
2. Cell conditioning scan
3. Cell wash
4. Buffer scan
5. Cell wash
6. Sample scan
7. Cell wash
8. Buffer scan
9. Final cell wash

2 Samples Each mg/mL

Nano DSC Applications

- **Biopolymer – Stability**
- **Biopolymer Structure**
  Domain, Subunit, oligomerization
- **Ligand Interactions**
  Drug Binding to Proteins or Nucleic Acids
- **Membrane Structure**
  Lipid Bilayers, Membrane proteins

- **Pressure Perturbation**
  structure and solvation
- **Complex milieu**



Dash = blood serum
Solid = pure proteins in serum

Types of questions addressed - Nano DSC

Stability of proteins and protein structural components

Why that linear sequence folds into *that* structure

design drugs that bind to 'diseased' protein

understand how proteins interact with and control each other, etc.

Cooperativity and reversibility of unfolding/folding reactions

Environmental effects on stability and reversibility

Enthalpic and entropic contributions to protein stability

Stability of molecular assemblies (e.g. liposomes)

Effect of ligand binding on protein-ligand complex stability

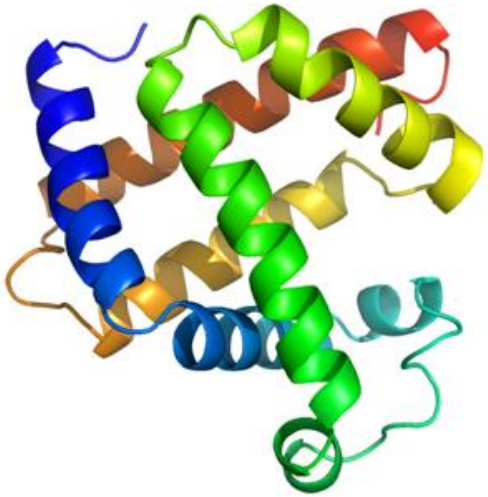
Experimental approaches are applicable to all biological macromolecules.

Why study proteins by DSC?

- Small changes in linear sequence (even one amino acid) alter 3-D structure, can profoundly change function (e.g., cause disease)
- Understand *why* that linear sequence folds into *that* structure
  - design drugs that bind to 'diseased' protein
  - design new proteins with new functions
  - understand how proteins interact with and control each other
    - and more!
- A protein 'structure' is actually an ensemble of very similar structures. Structures are constantly fluctuating slightly. Protein structures are dynamic
- Folding and structure are controlled by kinetics and thermodynamics.
- DSC is direct: no immobilization, no chemical derivatization

Understanding quantified values from a DSC

What stabilizes a protein structure?



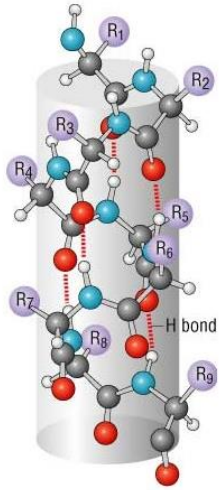
Free energy required to denature a protein is ~ 0.42 kJ/mol per amino acid.

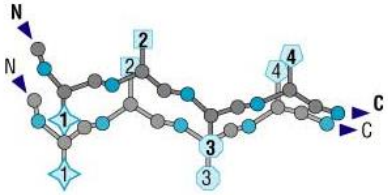
100 residue protein is stabilized by about 42 kJ/mol

Interactions with the environment (salts, membranes, ligands, other proteins) are critical to the structure of a protein.

Levels of protein structure

Magnitude of enthalpy corresponds with level of structure and type of folding (β-sheet, α-helix)

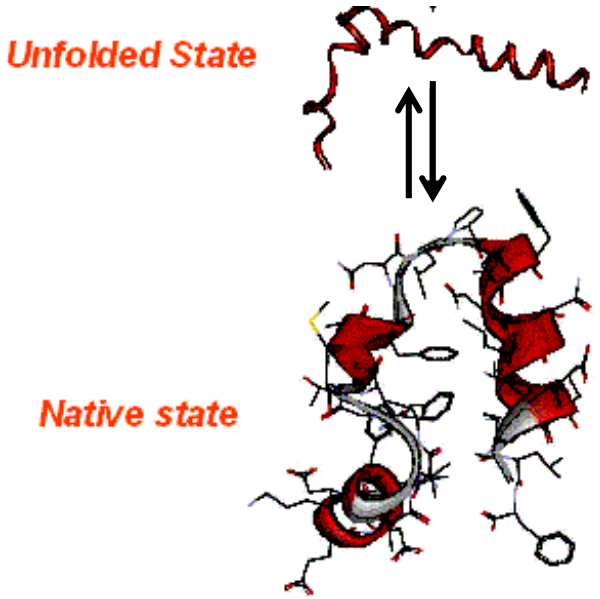




What happens to a protein during a DSC scan?

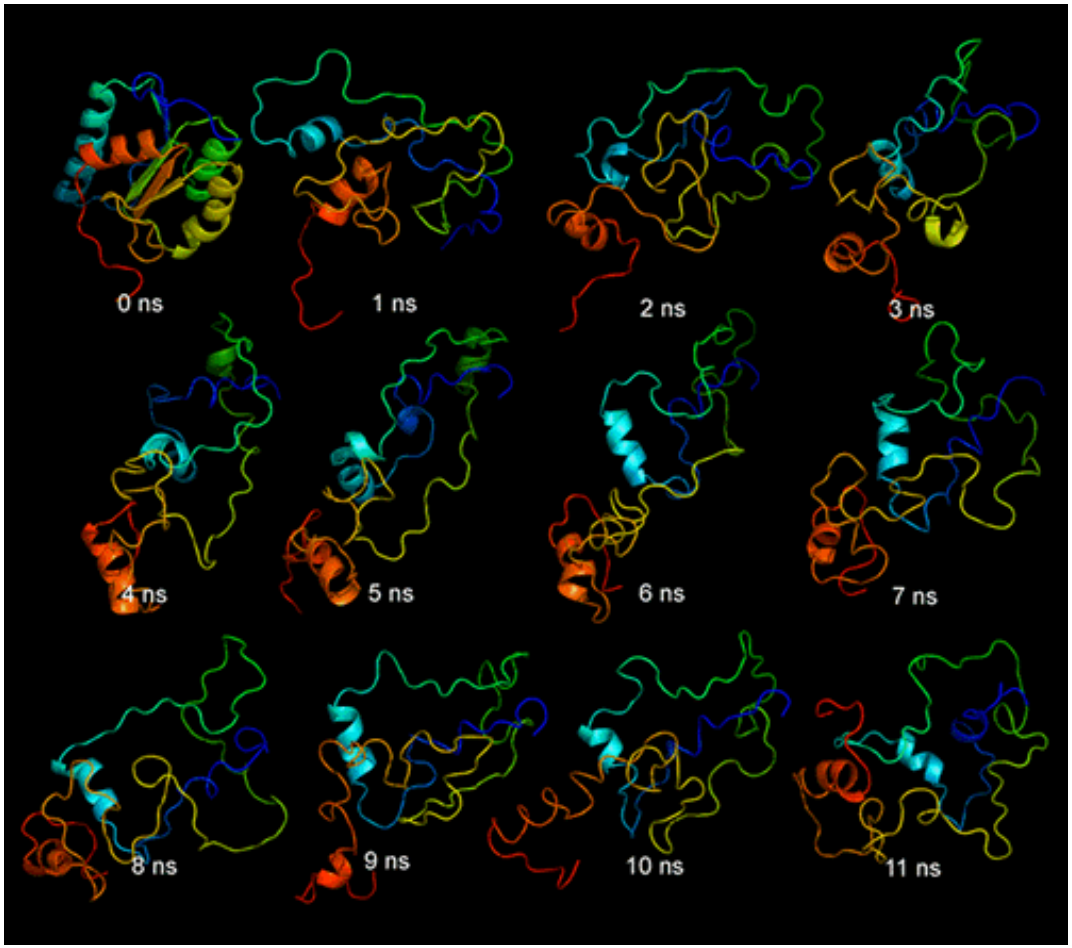
- The moderate heat used in a DSC scan will break the very weak bonds and interactions that stabilize the three dimensional structure.
  - Heat absorption causes the protein atoms to vibrate and move, disrupting stabilizing bonds and interactions. Hydrophobic groups are exposed to water. Proteins stick together and precipitate.
- Most proteins unfold between 40 – 90 ^{o}C
  - Temperature of unfolding (T_m) of a protein is characteristic for that protein

MET LEU SER ASP GLU ASP PHE LYS ALA VAL PHE GLY
MET THR ARG SER ALA PHE ALA ASN LEU PRO LEU TRP
LYS GLN GLN ASN LEU LYS LYS GLU LYS GLY LEU PHE



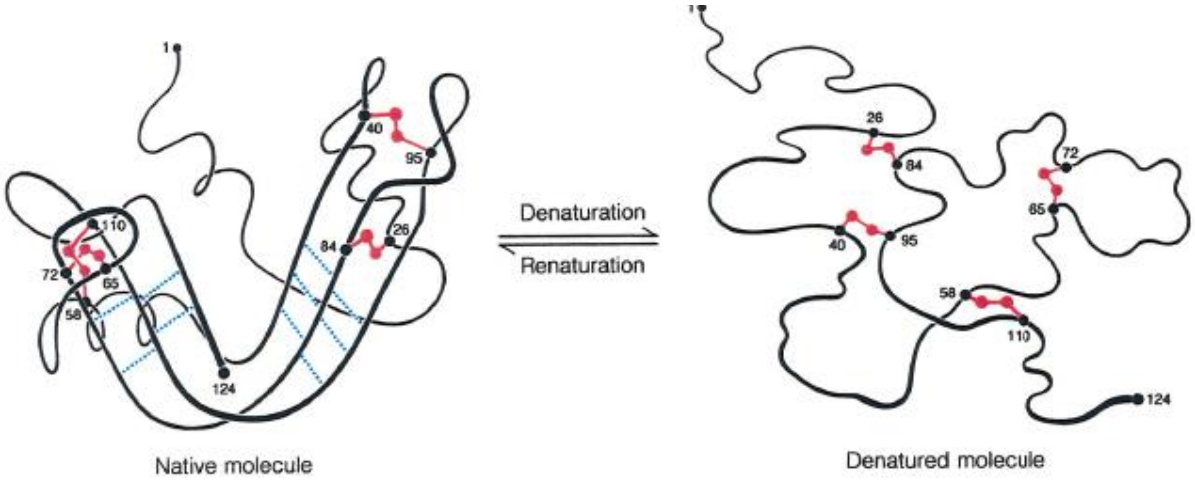
What happens to a protein during a DSC scan?

- The linear sequence folds into a complex 3-D structure with millions of very weak bonds and interactions
- Protein Stabilized by about 80 – 120 kJ/mole.
- Proteins unfold (denature) easily (change in pH, temperature, salts, organic solvents)



Heat absorption → vibration → disrupting stabilizing bonds and interactions

Two state model of protein unfolding



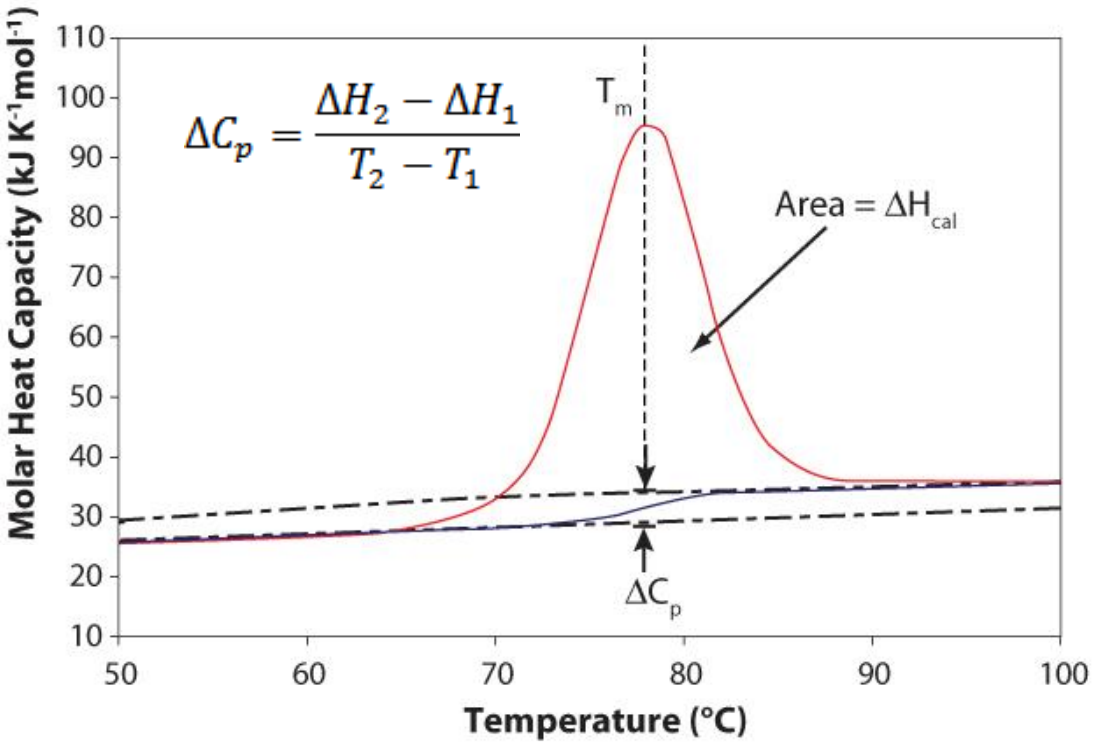
Enthalpically Favorable
Hydrophobic & electrostatic interactions, H^+ bonding

Entropically Favorable
Changes in solvation & conformational freedom

- Heat associated with unfolding (endothermic) and folding (exothermic) is easily measured by calorimetry, allowing thermodynamic analysis of the folding/unfolding process.
- Folding and unfolding of a small protein, a domain, or a subunit, is ‘cooperative’ (once started, it goes to completion).
- These small units can fold and unfold reversibly. Reversibility is directly measurable using DSC.

Information from DSC

- **Heat capacity change (ΔC_p)**
  - Trend: Protein with lower ΔC_p - more rigid and protein with higher ΔC_p - more flexible.
  - Primarily reflects exposure of hydrophobic groups. ΔC_p is positive.
- **Enthalpy**
- Δ H: e^2 non-covalent interactions – hydrophobic & electrostatic, H+ bonding
- **T_m**
  - Indicates macromolecular stability
  - From these measured values it is possible to calculate the entropy (ΔS) and free energy (Δ G)



ASTM E2603-08 - for verification of enthalpy and temperature of a fixed-cell DSC (www.astm.org)

What does a DSC scan tell us?

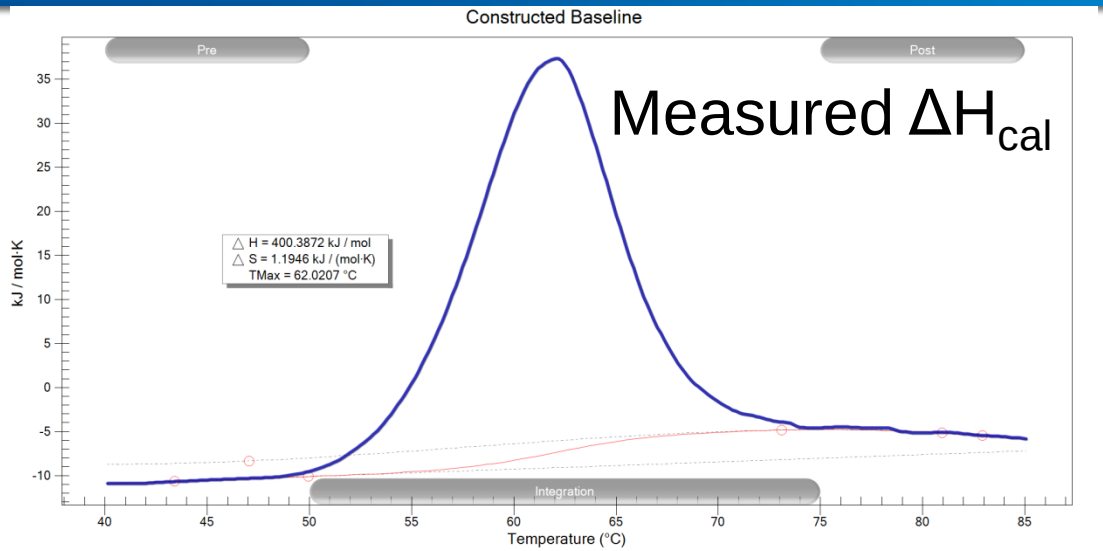
- If the native (N) and unfolded (U) state are in equilibrium
  - Assume Two-state

$$N \rightleftharpoons U$$

  - At any T:
    - $K_{eq} = [U] / [N]$
    - $\Delta G = -RT \ln K_{eq}$
    - $\Delta G = \Delta H - T\Delta S$

- At T_m, ½ unfolded, $\Delta G = 0$ then $\Delta S = \Delta H/T_m$ and [N] = [U]
- The measured values, are used to calculate entropy (ΔS; a measure of molecular disorder) and free energy (ΔG).
- Free energy describes the overall stability of the system.
- Positive ΔG: the folded protein is more stable than the unfolded protein.

Enthalpy

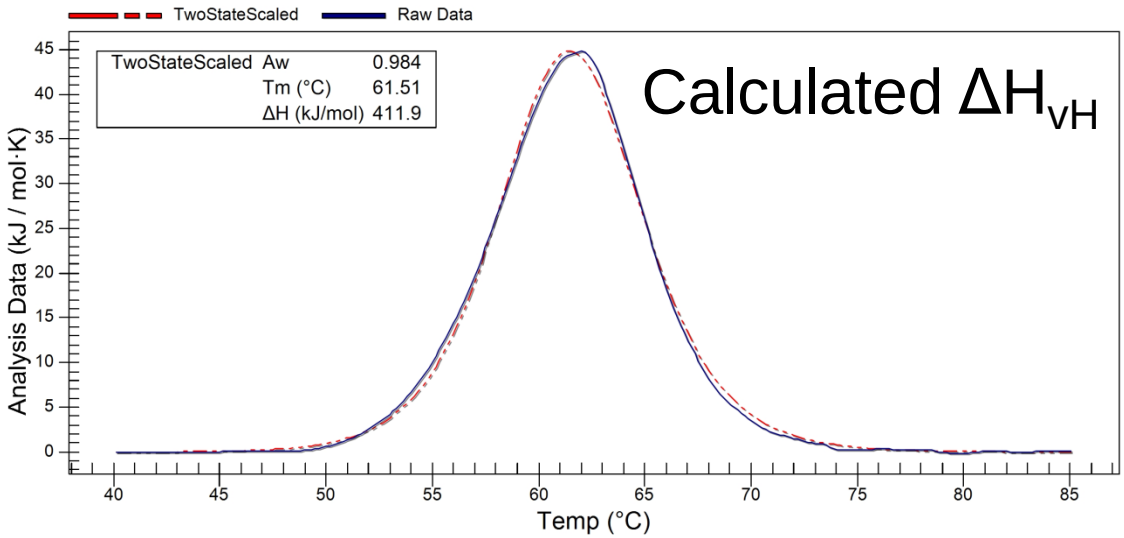


Measured ΔH_{cal}

T_m (ΔG) comes from two places: ΔH and ΔS.

Enthalpy

ΔH: e^2 non-covalent interactions – hydrophobic & electrostatic, H+ bonding



Calculated ΔH_{vH}

Enthalpy: Comparison of measured (ΔH_{cal}) and calculated (ΔH_{vH})

$$N \underset{}{\overset{k_1}{\rightleftharpoons}} D$$

$\Delta H_{vH} < \Delta H_{cal}$	$\Delta H_{vH} = \Delta H_{cal}$	$\Delta H_{vH} > \Delta H_{cal}$
$N \leftrightarrow I \leftrightarrow U$,	$N \leftrightarrow U$	$N_n \leftrightarrow nU$ (oligomer)
$MW_{Co\text{-}operative\ unit} < MW_{protein}$	$MW_{Co\text{-}operative\ unit} = MW_{protein}$	$MW_{Co\text{-}op.\ unit} > MW_{protein}$
Two-state model invalid	Two-state model valid. Cooperative unfolding	Two-state model valid or process is irreversible – hysteresis

Privalov and Potekhin "Scanning Microcalorimetry in Studying Temperature-Induced Changes in Proteins" Methods in Enzymology (1986), v**141**, pg4-51

$\Delta H_{vH} < \Delta H_{cal}$

Do two portions unfold independently?

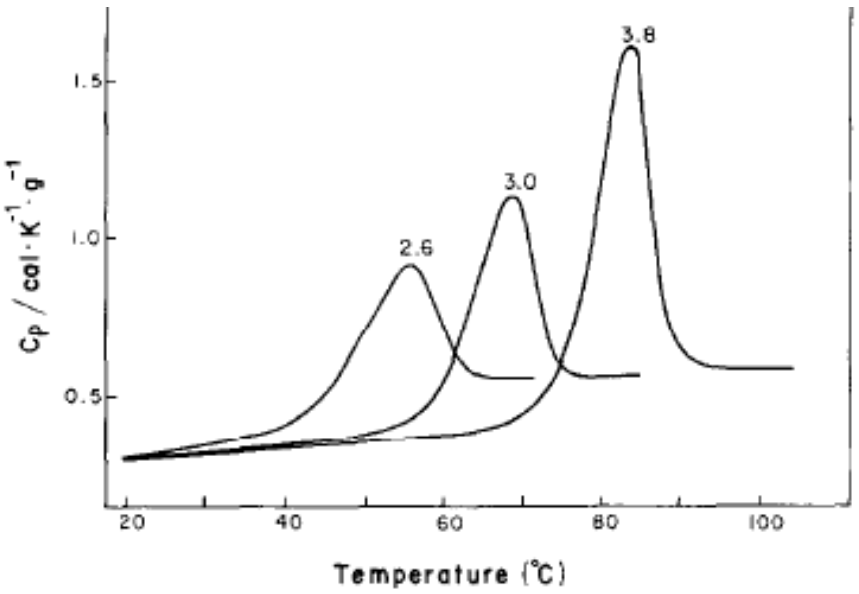


Fig.1. Temperature dependence of partial specific heat capacity of papain in solutions with different pH.

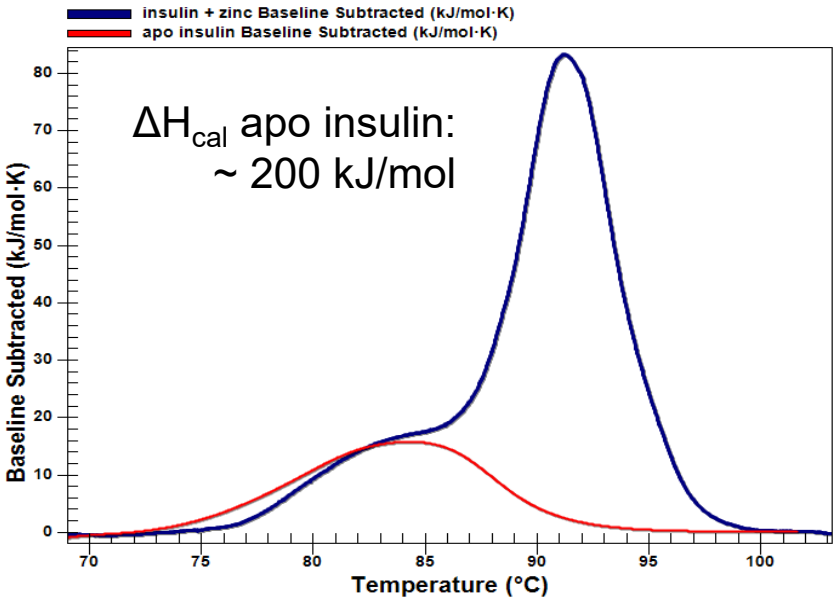
Increase of protein stability and the heat absorption peak also becomes sharper

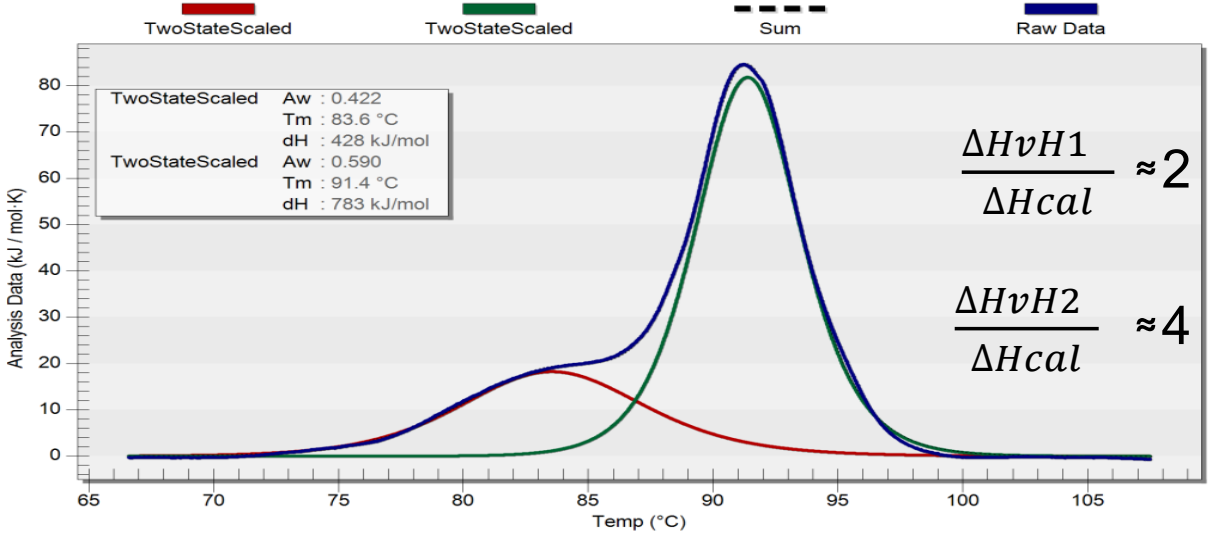
This sharpness can be quantitatively evaluated with ΔH_{VH} . $\Delta H_{vH} < \Delta H_{cal}$

Quasi-independent cooperative. Ratio is ~1.7 (ΔH_{cal}/ ΔH_{vH})

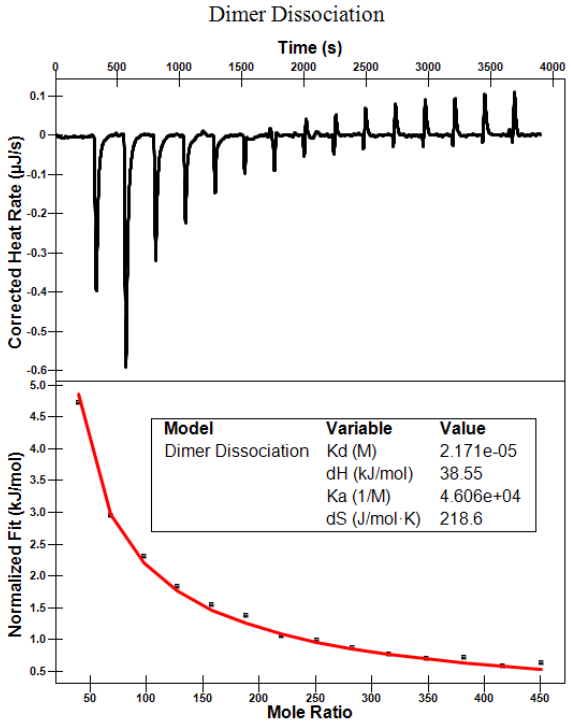
Privalov, Microcalorimetry of Macromolecules, 2012 Wiley

$\Delta H_{vH} > \Delta H_{cal}$





Confirm dimer formation with ITC



K_d = 22 µM
DSC [sample] > 100 µM

Entropy and Heat Capacity

- ΔS: an expression of the multiplicity of ways in which the system can be found with a particular energy
  - Degeneracy (w) of the system.

- ΔC_p relationship with ΔS. $\Delta C_p = T \frac{\delta \Delta S}{\delta T}$

- C_p depends on the numbers of ways there are of distributing any added heat energy to the system

  - Because ΔC_p is easier to visualize than ΔS from a DSC thermogram, the focus will be on this property

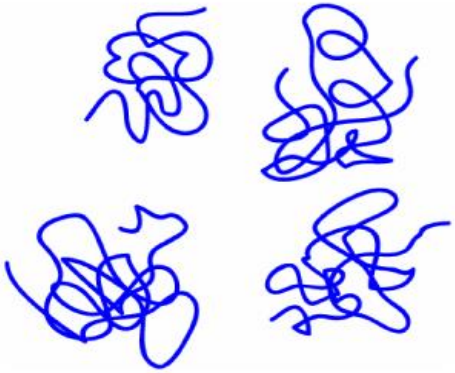
  - ↓ w and ↓ S then ↓ C_p, little energy will be required to raise the temperature

Entropy and Heat Capacity

- S: an expression of the multiplicity of ways in which the system can be found with a particular energy.
  - Degeneracy (w) of the system.

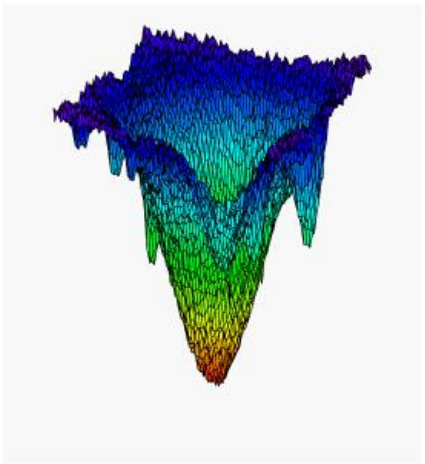
$$\Delta C_p = T \frac{\delta \Delta S}{\delta T}$$

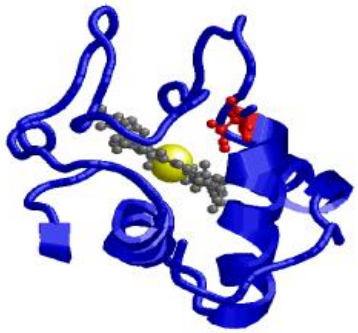
- C_p depends on the numbers of ways of distributing added heat energy.



Unfolded states

An astronomical number of conformations. A 100 residue protein, with 2 conformations per residue has 2^{100} or 10^{30} different conformations





Folded or Native State

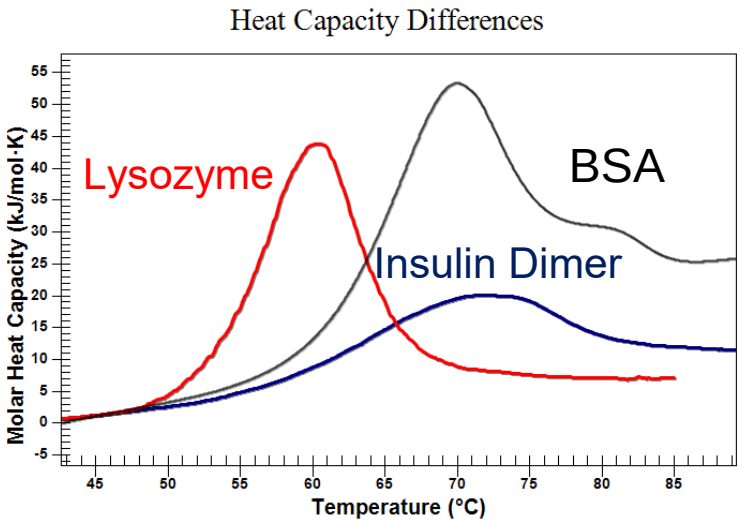
A single conformation (or, more correctly, a collection of similar conformational sub-states)

Cartoon: http://www.lanl.gov/bmsi/Individual%20Research/Werner/WernerFolding.html

Heat Capacity

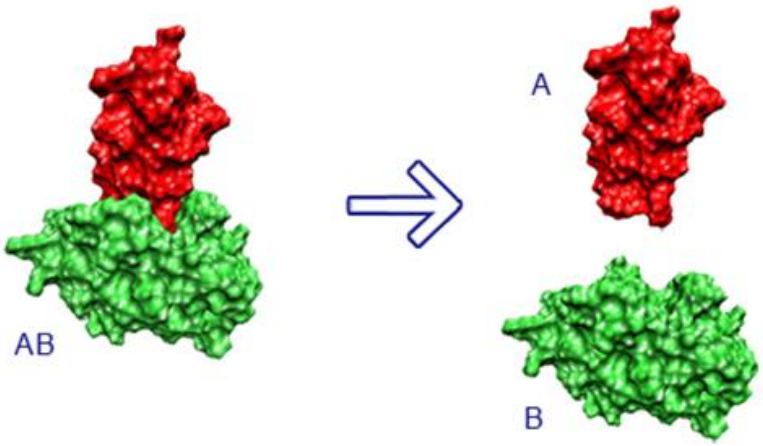
- $+\Delta C_p$: typically a denatured protein has a higher heat capacity than the native folded protein.

- Reasons for $+\Delta C_p$
  - Solvent reorganization around newly exposed non-polar side chains.
  - ΔC_p scales with the number of buried hydrophobic side chains in the native conformation.
  - Major influence of C_p comes from the primary sequence, minor influences are non-covalent
  - Gomez et al. *Proteins*, 1995, 22, 404
  - "Iceberg" theory. There is a cage of water that forms around the non-polar groups that is said to contribute to the large heat capacity. Sturtevant, PNAS, 1977, 74, 2236

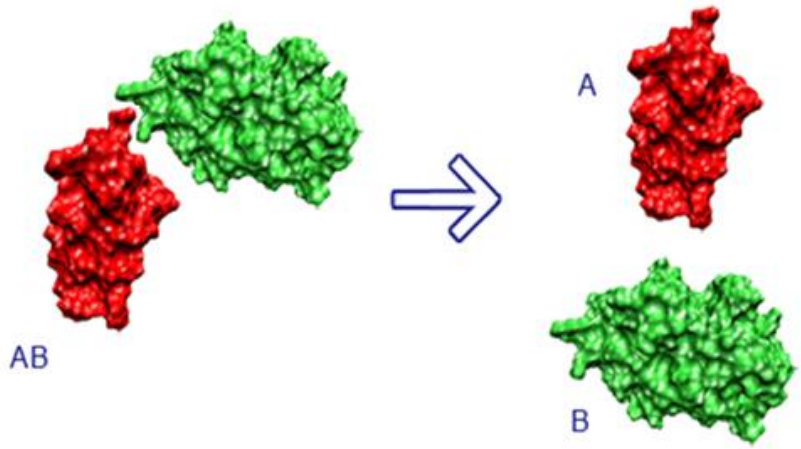


Heat Capacity & Binding

Large, + ΔC_p



Smaller, + ΔC_p



ΔC_p is directly related to solvation

In case of L binding to P, if ΔC_p <0 (reverse direction of above), this is associated with ligand-induced structural changes that bury hydrophobic resides and liberate solvent (JACS (1964), 86, 4302)

DSC Analysis Summary

- T_m
  - Stability
  -
- Heat Capacity
  - Degeneracy
  - Excess molar C_p following unfolding and longevity
- Enthalpy
  - Van't Hoff relationship
  - Cooperativity

Next step is to apply this analysis to an actual samples

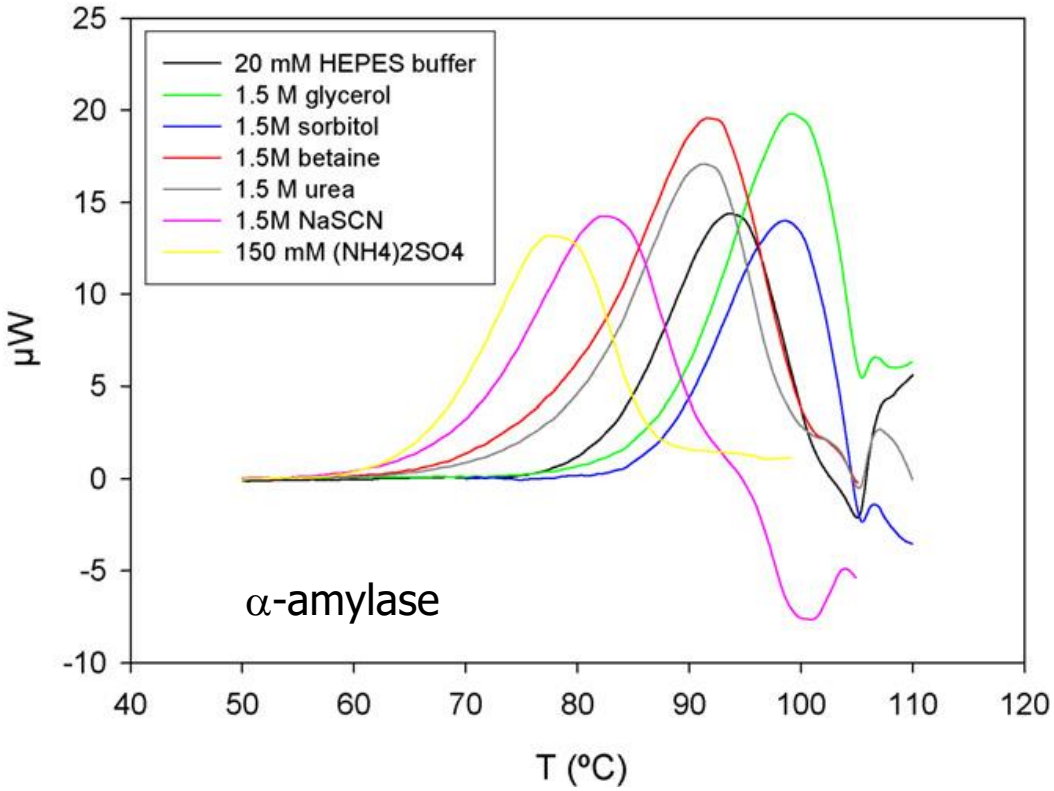
Applications

Applications for DSC

- Determine thermal transition (melting) temperatures
- Measure ΔH of denaturation
- Measure reversibility of thermal processes
- Measure ΔC_p of unfolding
- Determine stability macromolecules
- Measure high affinity binding (up to 10^{20} M^{-1})
- Investigate a complex milieu

Effect of additives and formulations

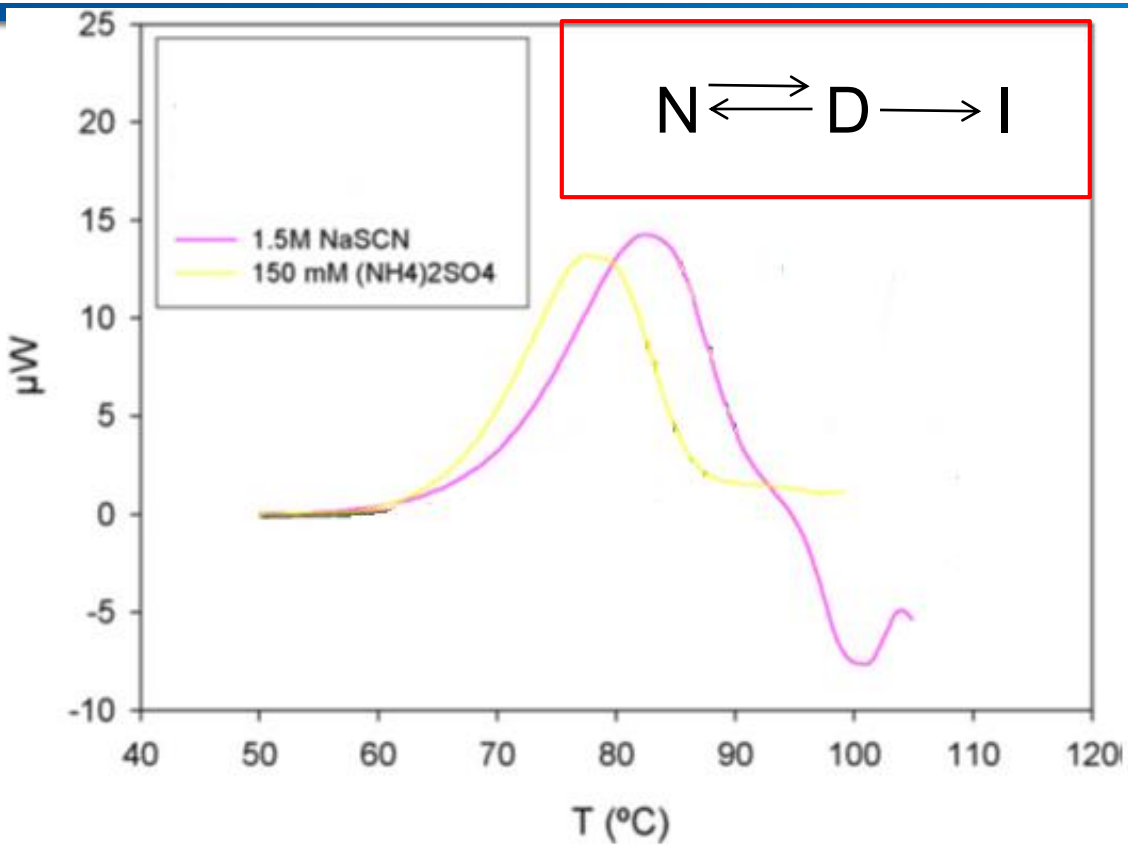
- Excipient to stabilize against chemical and physical degradation
- Choice of an additive or a formulation is generally determined empirically
- DSC is the fastest way of evaluating additives effect on T_m, reversibility



Olsen et al., *Thermochimica Acta*, **484**, (2009), 32-37

Many users stop data analysis after reporting a T_{max}, the following slides will discuss what is being missed.

Denaturation and Aggregation



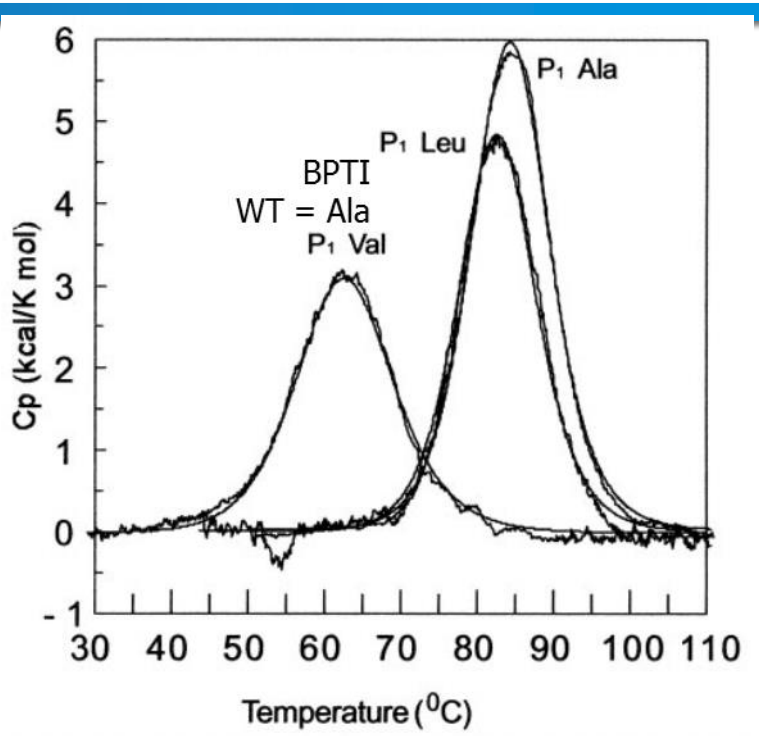
- Is the NaSCN trace a better choice than $(NH_4)_2SO_4$?
- Correlation of proteins with aggregation tendencies to poor longevity, using real-time HPLC SEC.

Techniques for studying denaturation and not aggregation:

1. pH far from isoelectric point
2. Dilute solutions
3. 3. Reversibility – above 80 ˚C mesophilic proteins typically not reversible b/c of oxidation of side groups

Macromolecular Stability

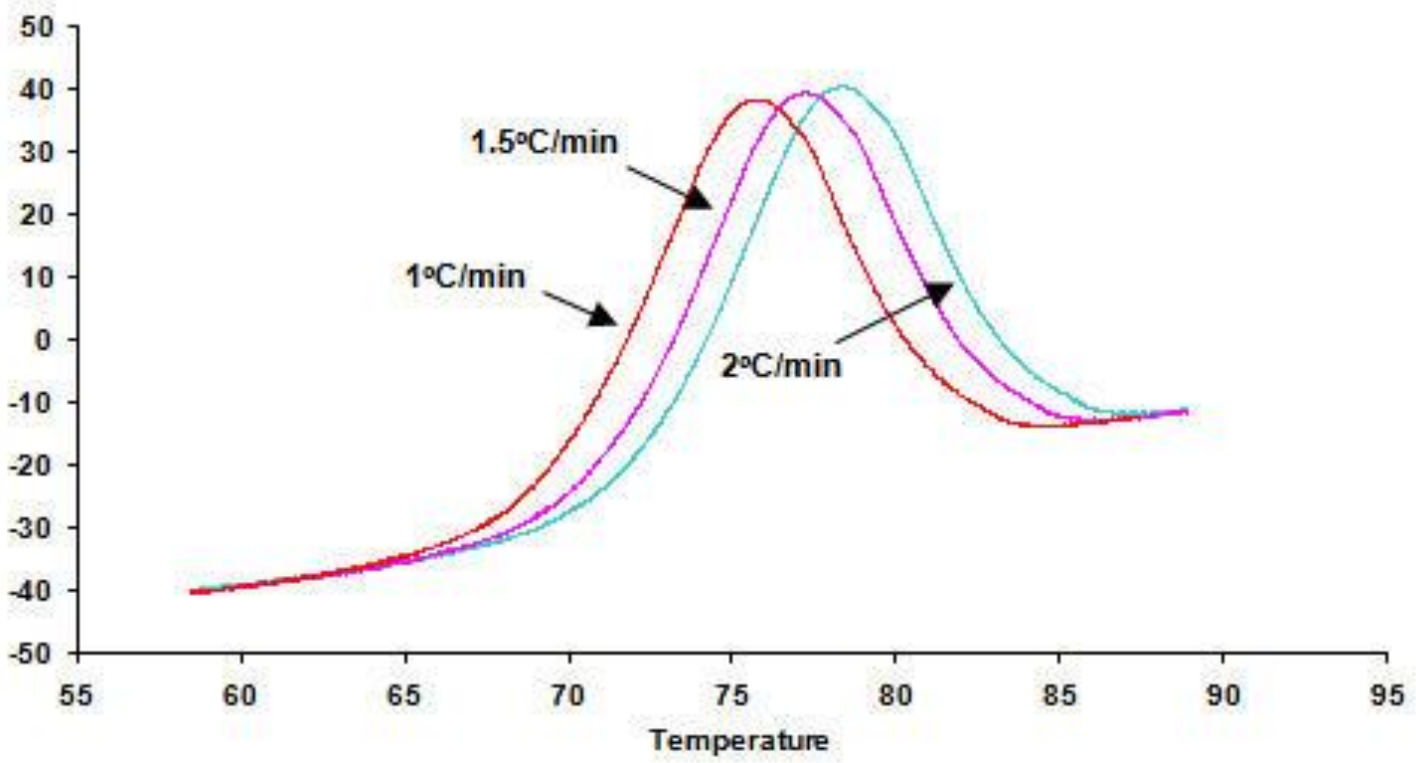
- Mutate proteins: more stable, more specific, faster, new properties, etc.
- Useful to predict outcome of a mutation, so need a database of thermodynamically characterized proteins
- Complicated network of interactions
  - Example: enthalpic changes (changes in hydrophobic interactions, hydrogen bonding, electrostatic interactions) are compensated by entropic changes (changes in solvation, conformational freedom)



Grzesiak et al., J. Mol. Bio. **301**, 205-217, 2000

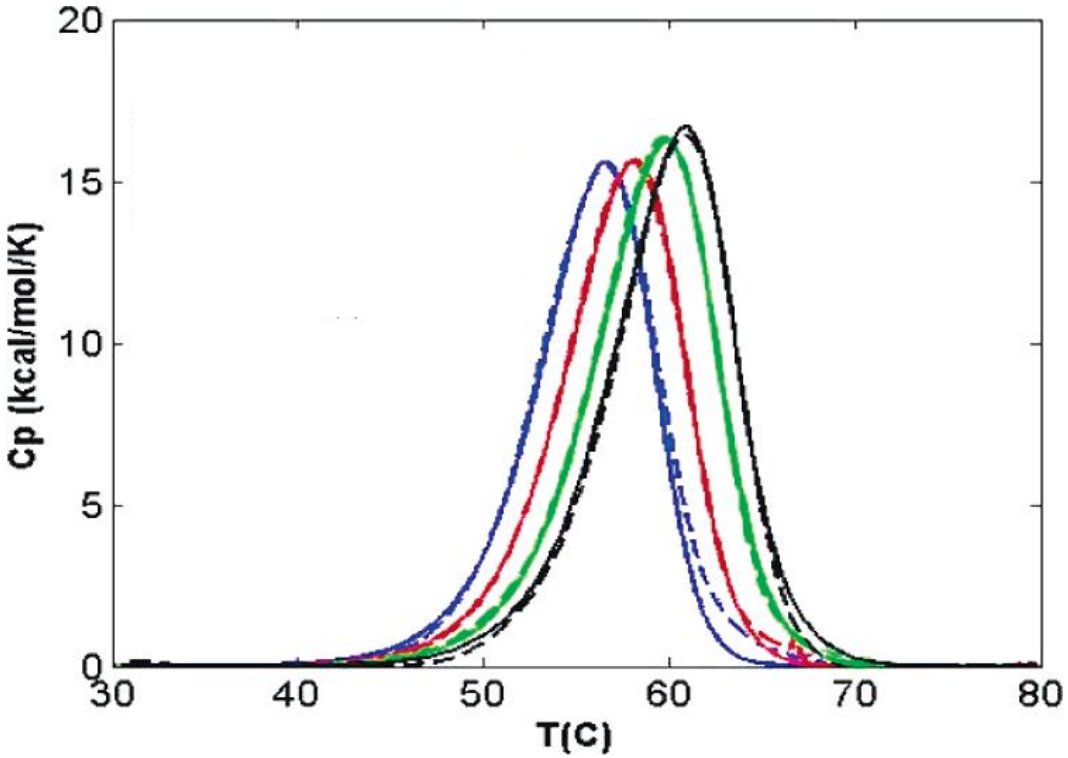
- Since $\Delta G = \Delta H - T\Delta S$, unfolding occurs when $T\Delta S$ increases sufficiently (e.g. by absorbing heat) to overcome stabilizing enthalpic interactions
- Biopolymer unfolding is endothermic

How to determine if unfolding is kinetically controlled?



- Scan rate dependence of T_m indicates that N and U are not in equilibrium.
- Their concentrations change at a rate equal to the sum of the unfolding and refolding reactions. Increasing temperature faster than system responds distorts T_m and shape.

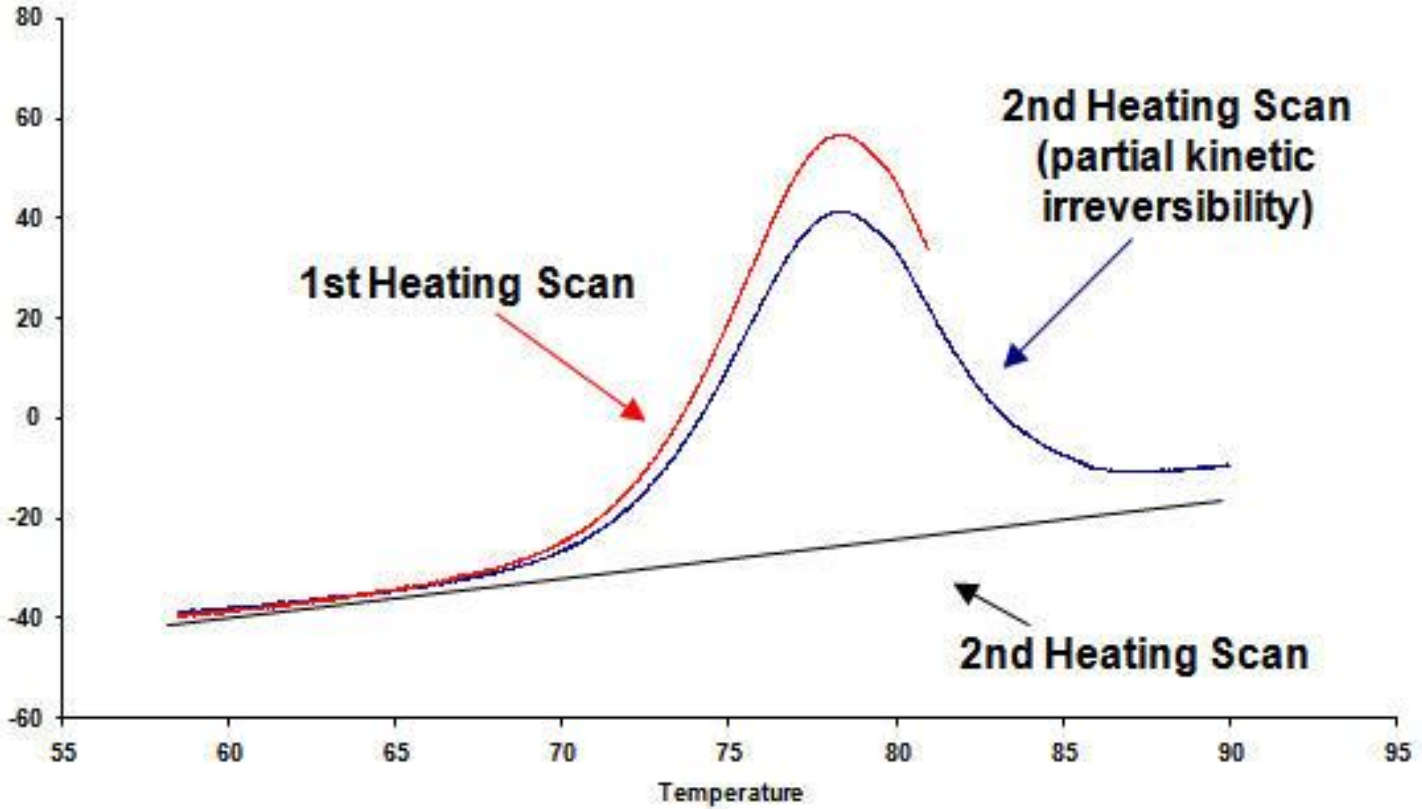
Protein unfolding/refolding reversibility - scan rate



Remmele et al., *JACS* **127**, (2005), 8328-8339

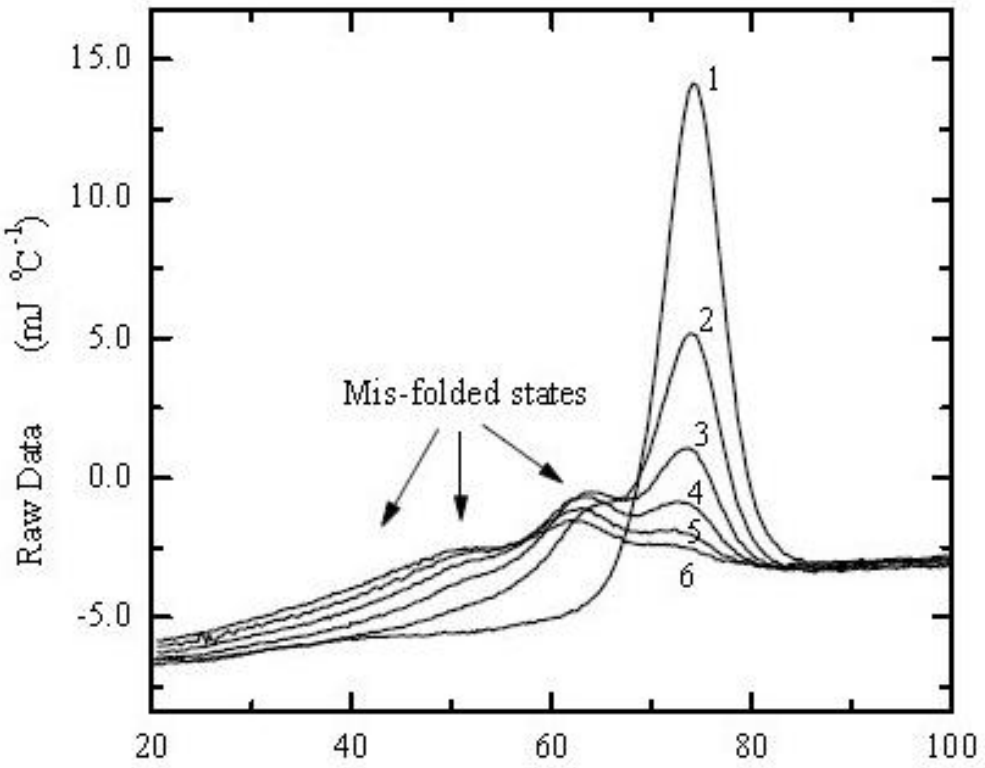
- Interleukin-1 receptor scanned at 0.25, 0.5, 1.0 and 1.5 ^{o}C/min:
  - Scan-rate dependence of T_m indicates folded and unfolded protein are not in equilibrium
  - Unfolding is kinetically controlled

How to determine if unfolding is reversible?



- Aggregation is characterized by thermodynamic and kinetic components.
  - Thermodynamic component causes unfolding.
  - Kinetic component can result in partial or complete irreversibility.

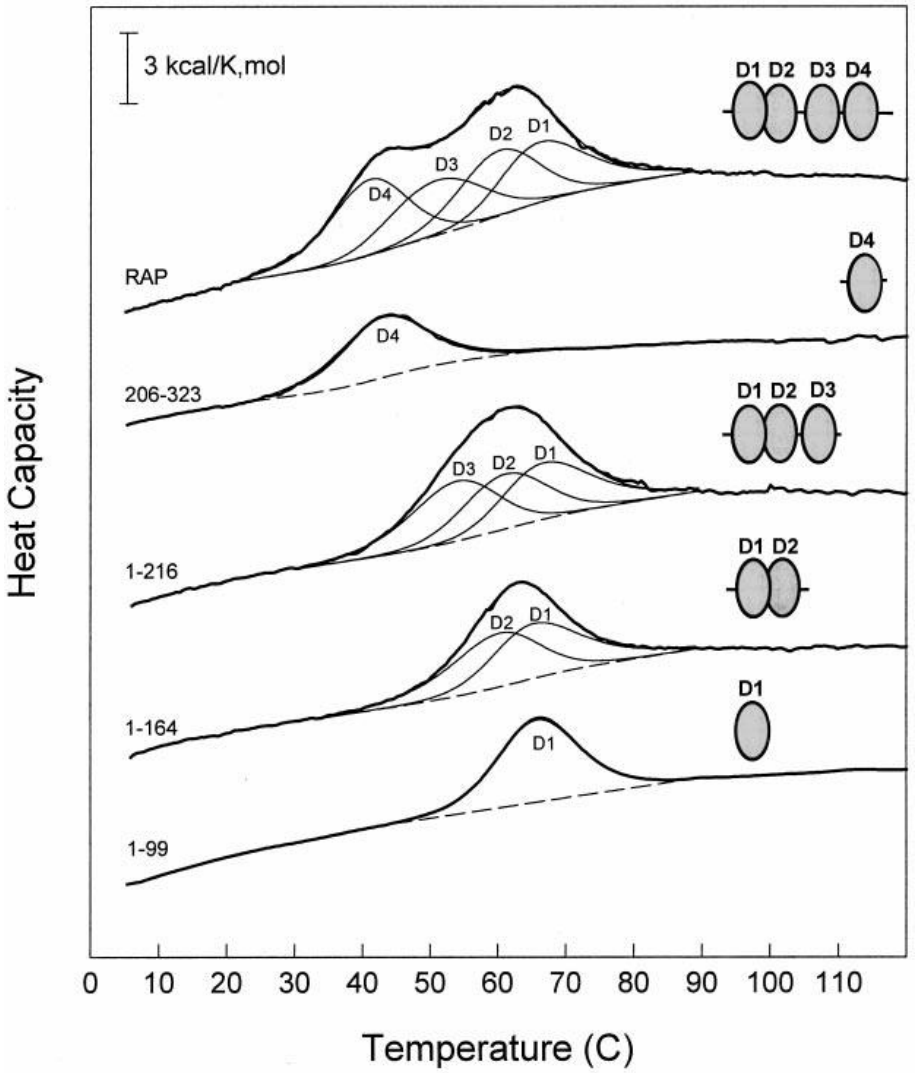
Protein unfolding/refolding reversibility - rescanning



- Non-reversibility of protein unfolding indicates:
  - Multi-domain or subunit structure
  - Chemical alterations to the sequence
  - Kinetic events hindering

1st heating scan to 6th heat scan shown

Domain and Subunit Stabilities

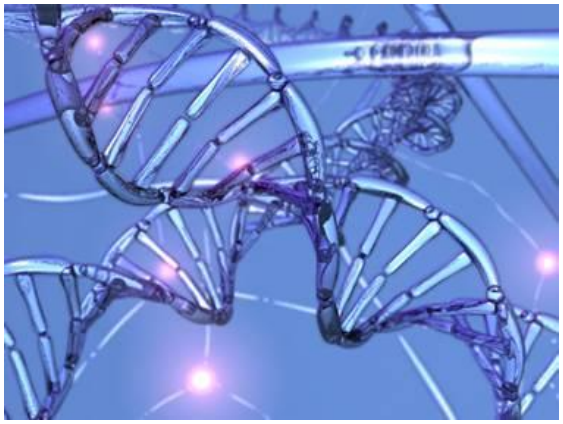


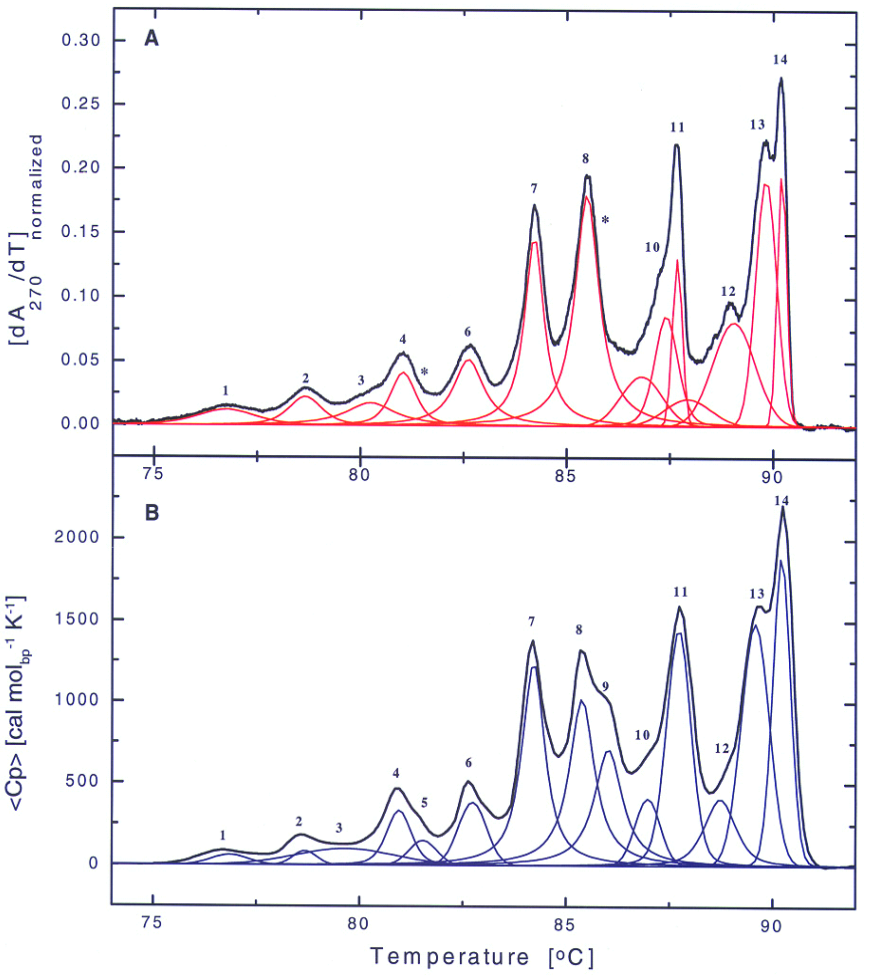
- Unfolding of domains and subunits with different thermal stabilities may produce asymmetric thermograms.
- A small change in sequence, or other alteration, can affect the stability of the whole protein, or the stability of one domain or subunit.
- DSC quickly reveals these stability changes.Practical implications: Identify subunit stability within a protein.

Medved *et al*., *J. Biol. Chem*. **274**, 717-727, 1999

Polynucleotide structure

- Linearlized pBR 322 heated at 0.1 ^{o}C/min for optical profile, 1 ^{o}C/min for DSC scan.
- Individual spectral peaks reflect the cooperative melting of one or more domains.
- DSC provides a complimentary and more direct means of measuring thermodynamic parameters of DNA.

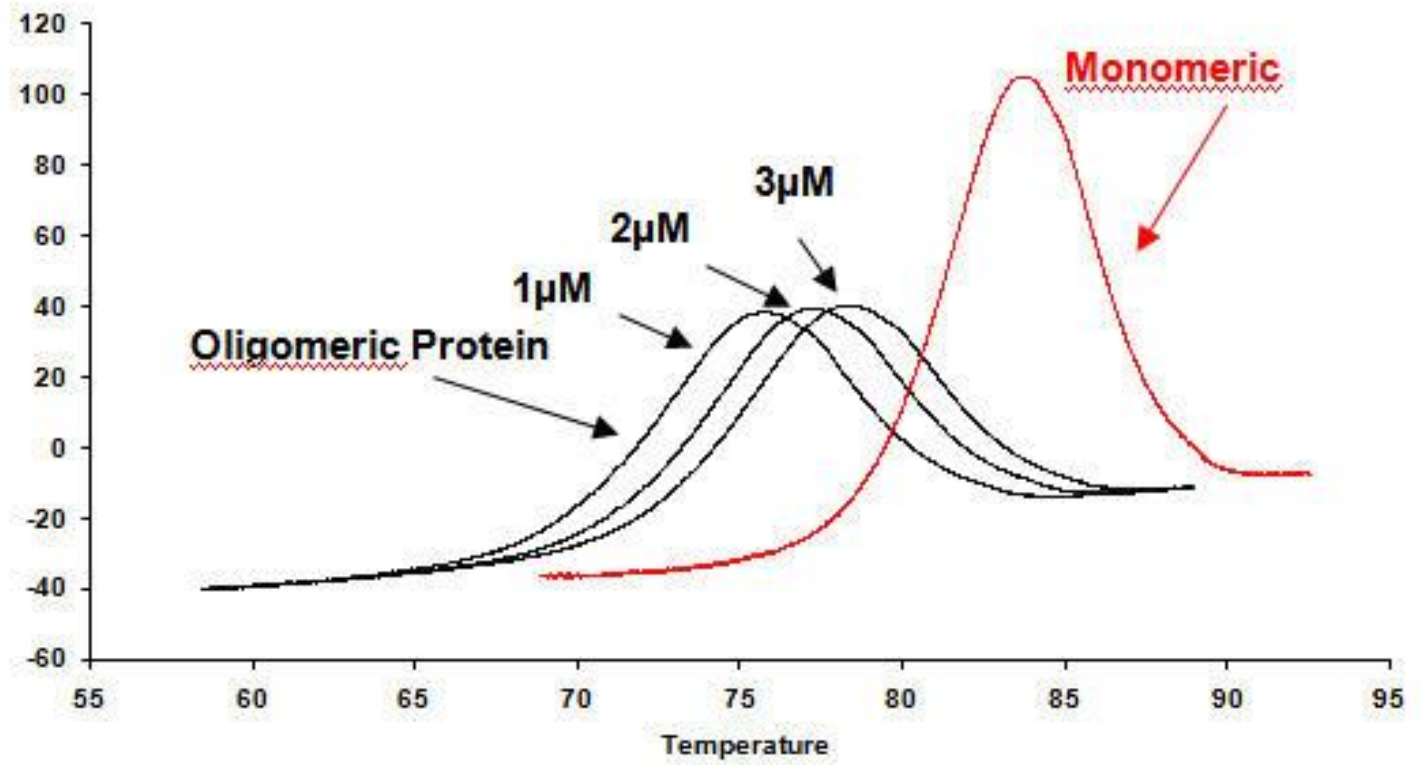




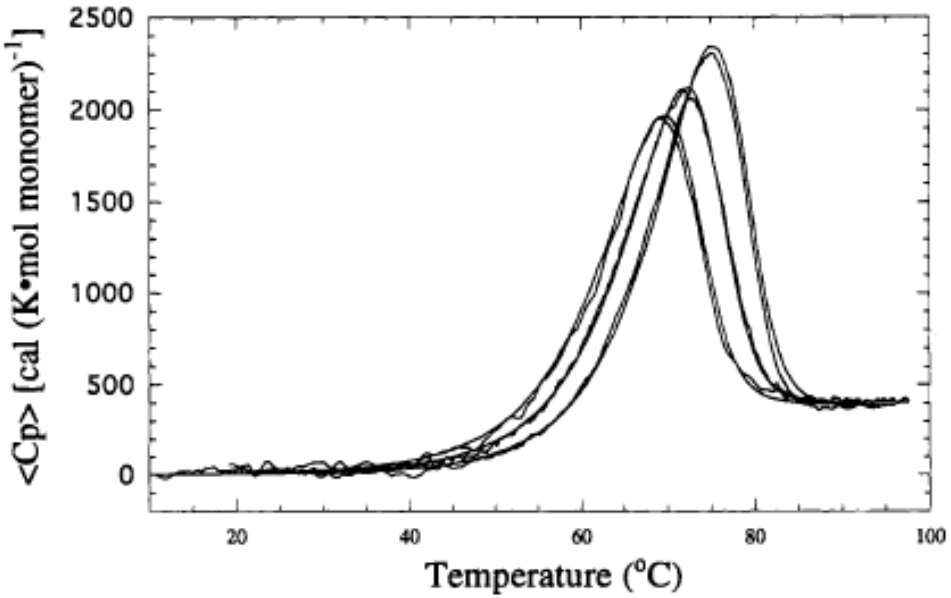
Volker *et al., Biopolymers* **50**, 303-318, 1999

Importance of concentration determination

Effect of sample concentration dependence of T_m is a test for oligomerization



Protein oligomerization

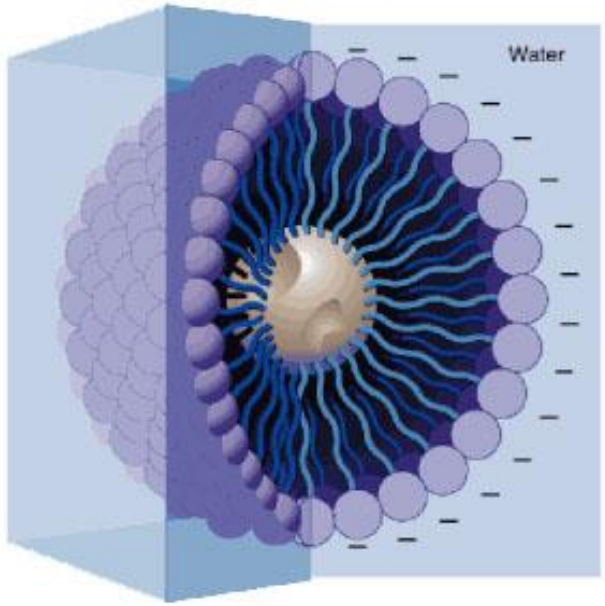


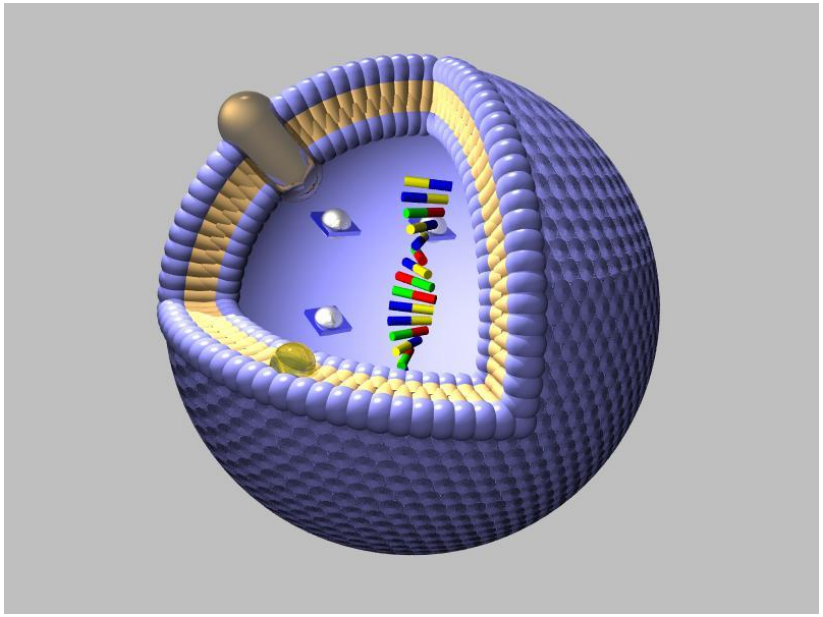
Johnson *et al., Biochemistry* **34** (1995) 5309-5316

- Oligomerization domain of tumor suppressor p53 studied at 70, 93 and 146 μM.
- T_m increase with increase dependent upon concentration demonstrates the formation of higher order association states

Membrane proteins and membranes: detergents and lipids

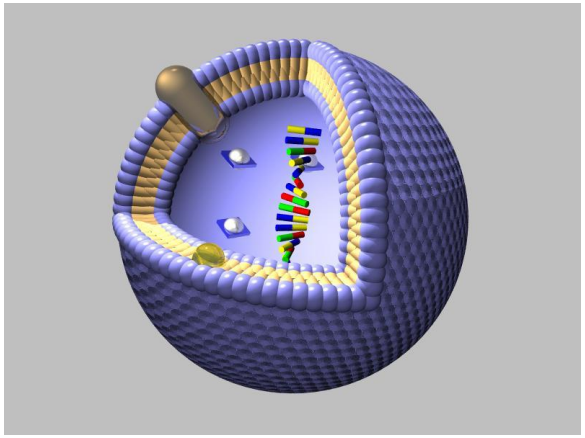
- Membrane proteins must be solubilized in detergents or lipids
- Detergents form nanospheres in water composed of a single layer of detergent molecules. This is called a micelle.
- Lipids form nanospheres in water composed of a double layer of lipid molecules, called a liposome.



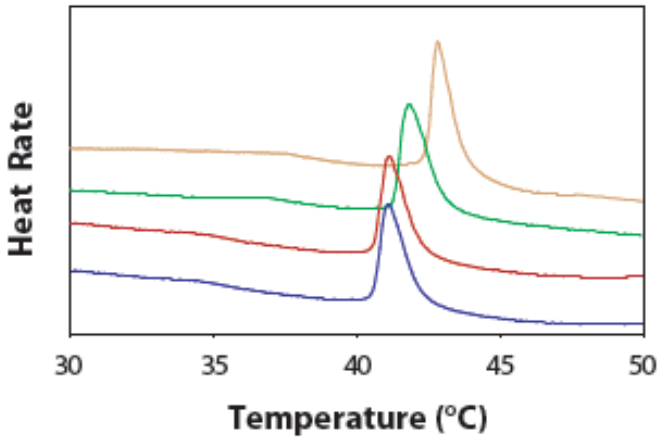


Membranes/Membrane protein Interactions

- Many proteins are associated with membranes and serve numerous functions.
- How does membrane stability change in the presence of various proteins
  - DSC can provide indications



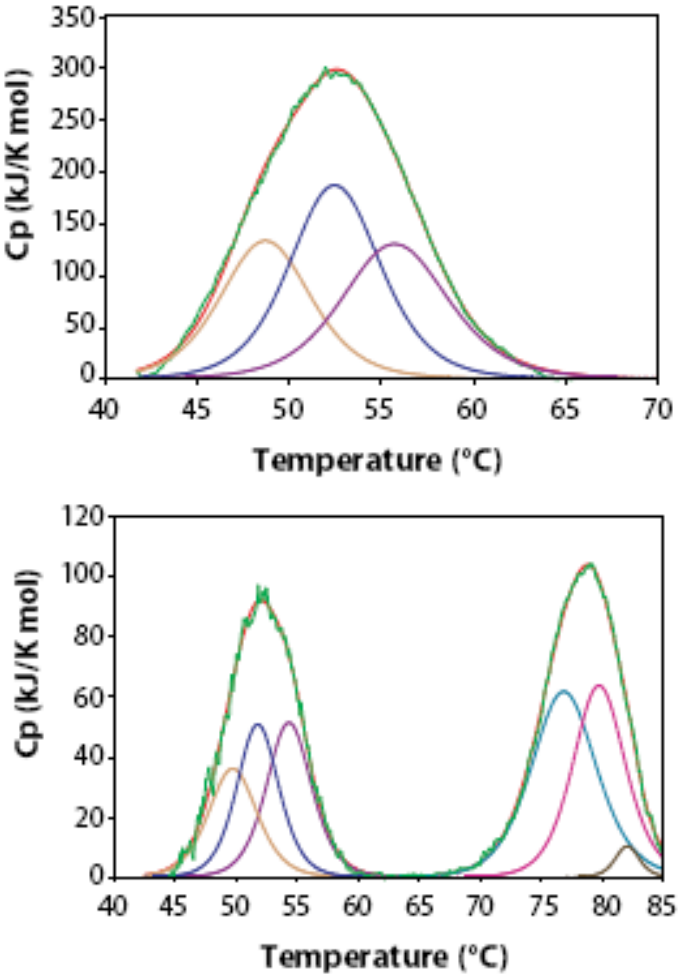
- Vesicles were prepared by sonicaton bath with 5 mg/mL DPPC and then treated
  - Palmitoylated SNAP-25 (Orange)
  - 1.2 µg SNAP-25 (Red)
  - 2 µg SNAP-25 (Blue)
  - Untreated control (Green)



Palmitoylated SNAP-25 increase membrane stability, while non-palmitoylated SNAP-25 decreases membrane stability relative to the control

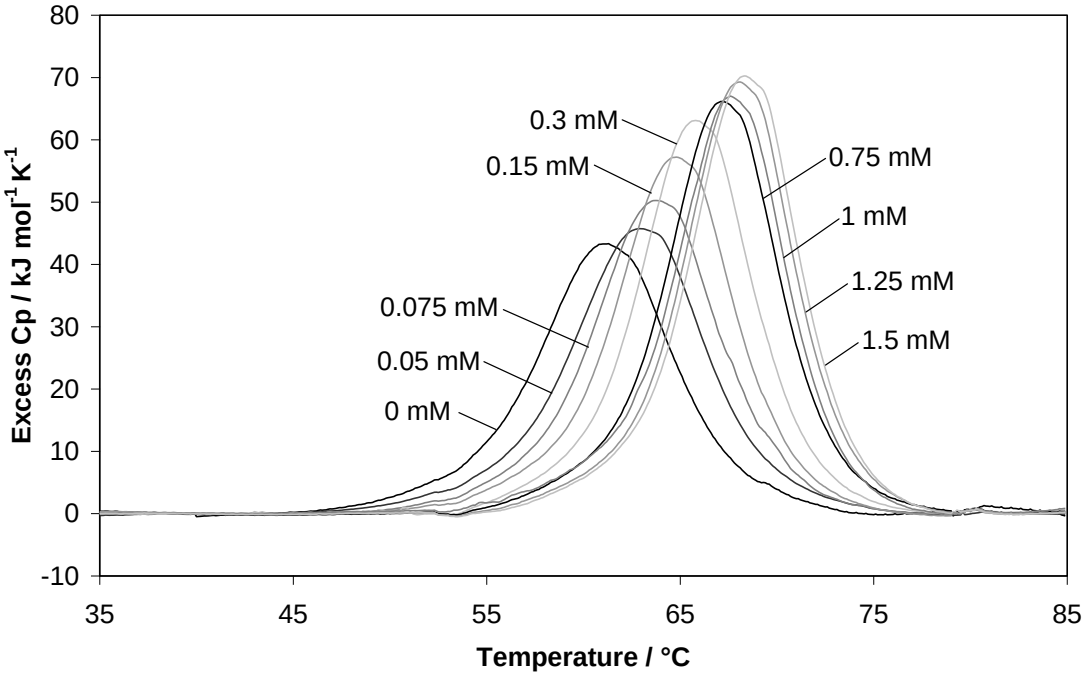
Membrane protein structure

- Micrograms of a membrane protein can represent weeks of work and require detergents for solubilization
- Nano DSC obtained high-quality scans using 20 mg (0.3 picomoles) of a 70,000 Da complex (non-palmitoylated and partially palmitoylated SNAP-25) consisting of 3 membrane proteins
  - Thermogram very well fit by 3 transitions
- Partial chemical derivatization and stabilization of complex easily verified by DSC



Studying Binding by DSC

- If a ligand binds preferentially to a folded protein, the T_m of the protein will generally increase. The more bound ligand there is, or the tighter it binds, the more T_m increases.
- Can determine binding constant at T_m.
- Useful for very tight binding
- DSC is a quick way to determine if two molecules interact.



Binding of 2'-CMP to RNase A ± 5% DMSO (black)
K_a = 5900 M^{-1} (-DMSO); 6900 M^{-1} (+DMSO) at T_m

Studying Binding Via DSC- Simplified

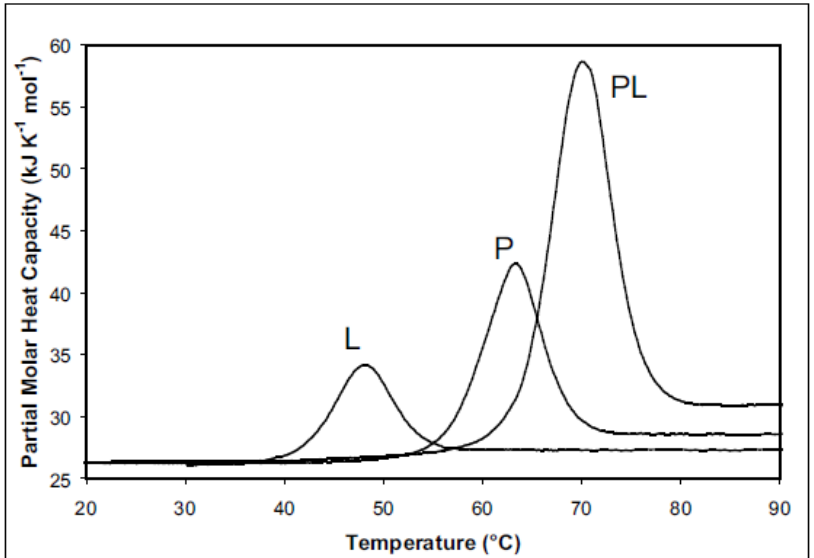


Fig. 2. Simulated DSC data showing the molar heat capacity of free ligand (L), free protein (P) and the protein-ligand complex (PL), where the complex is a tightly-associating system.

Advantages over other Techniques:

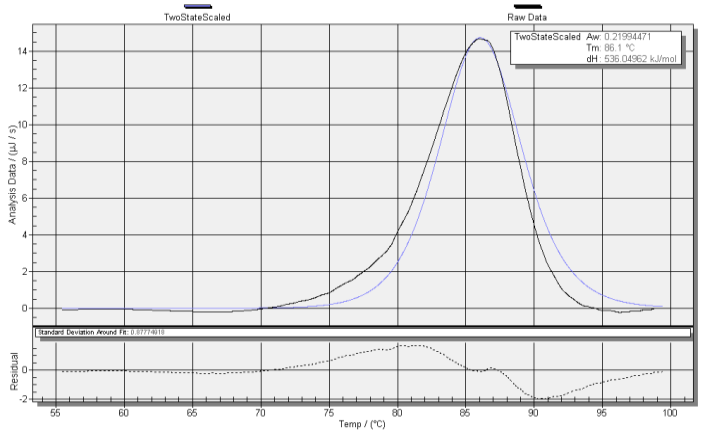
1. Ligand only soluble in organic solvents
2. Extremely tight binding affinities
3. Slow binding

$$K_a^{Tm} = \frac{\exp\{\frac{-\Delta H_{D-N}}{R}(\frac{1}{T_m}-\frac{1}{T_0})+\frac{\Delta C_{pD-N}}{R}(\ln(\frac{T_m}{T_0})+\frac{T_0}{T_m}-1)\}-1}{[L]}$$

Protein-ligand complex

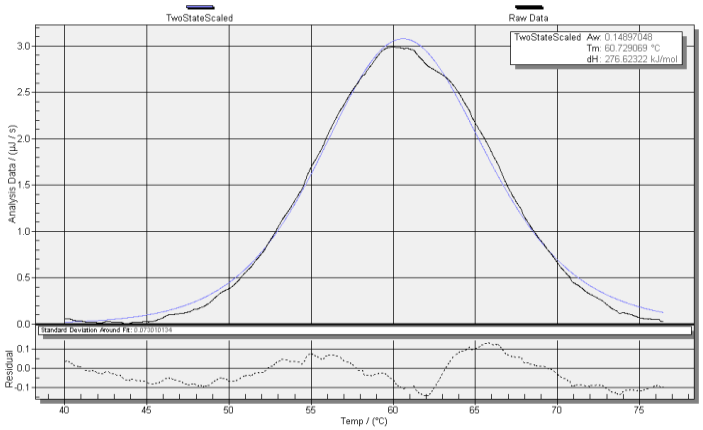
Nano DSC Scans from 0° C - 110° C at 1° C/minute

Nucleic Acid – DSC Scan



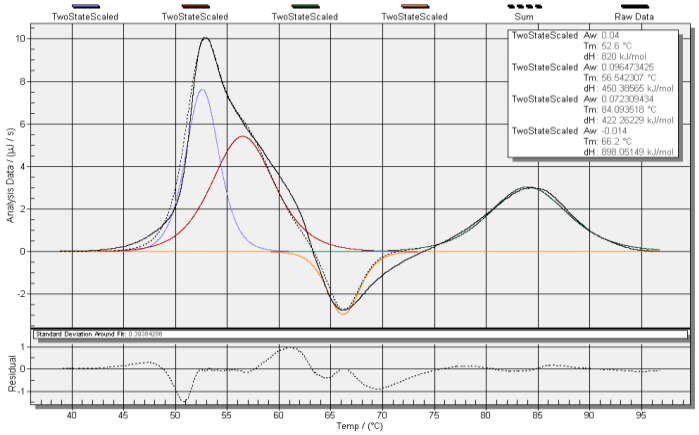
Tm = 86.1° C

Nucleic Acid-Binding Protein – DSC Scan



Tm = 60.7° C

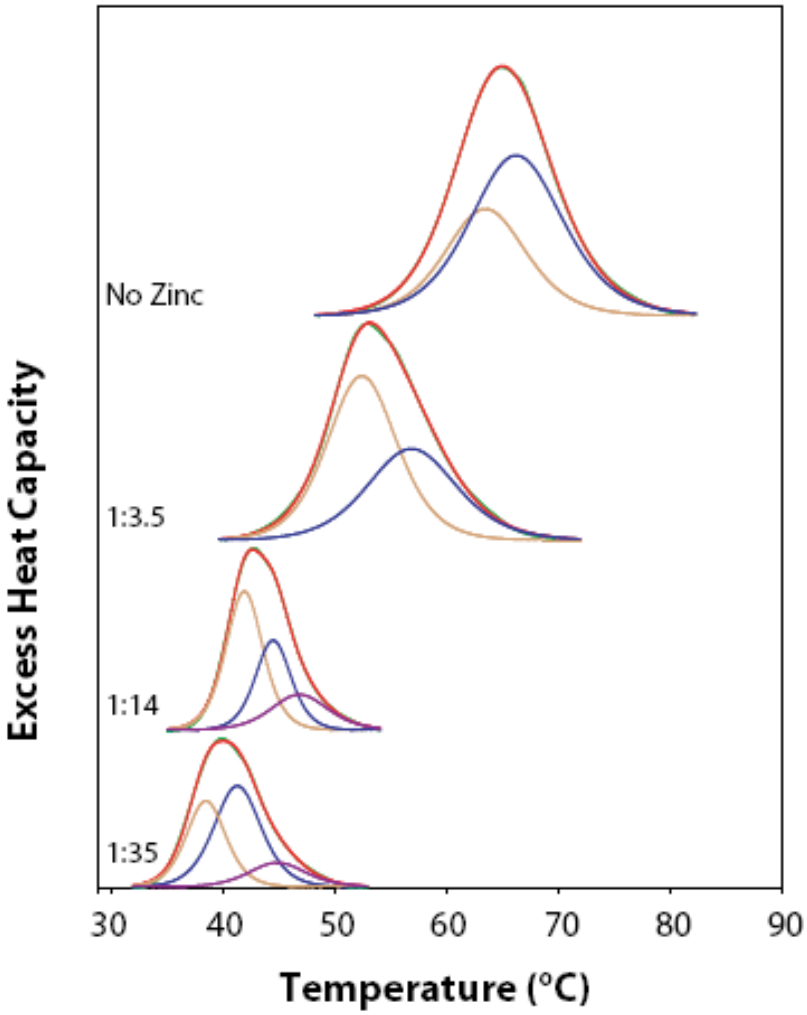
Complex – DSC Scan



Nano DSC analysis of the complex reveals several surprising details

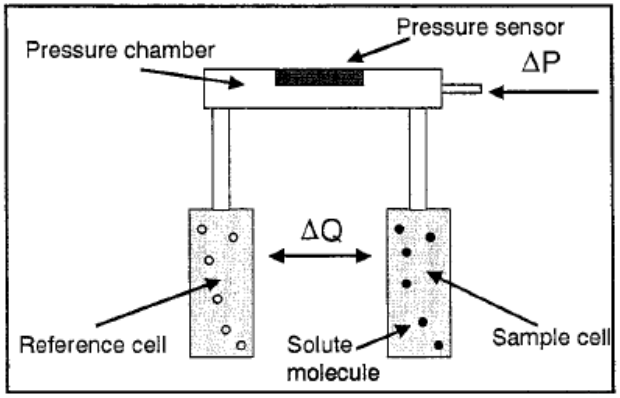
Tertiary structural changes upon ligand binding

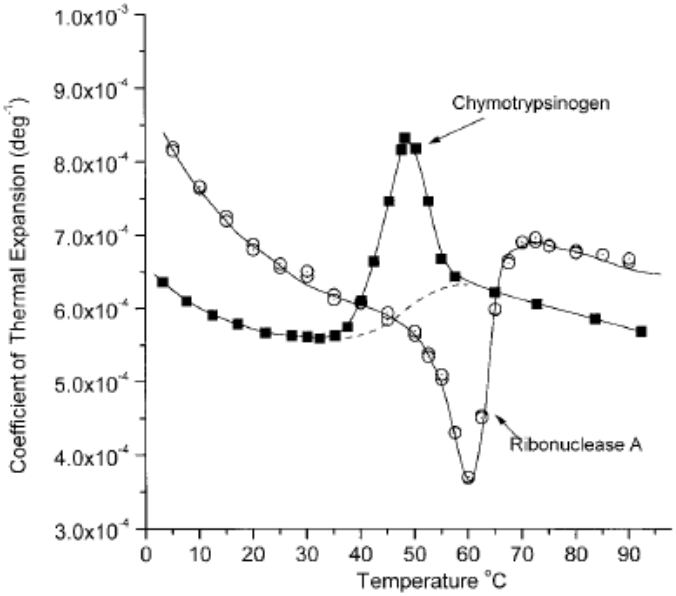
- Protein structures are dynamic and constantly fluctuating between partially folding and folded structures
- Ligands can preferentially bind to a partially unfolded conformation
- Ex: Zn^{2+} added to Ca^{2+}-saturated a-lactalbumin, a two-domain protein
- Enthalpies of the domains in absence of Zn^{2+} are consistent with the crystal structure
- Increasing [Zn^{2+}] progressively destabilizes the protein, driving equilibrium towards the unfolded state
- DSC is a quick way of determining if a ligand binds preferentially to folded or partially unfolded protein.



Pressure Perturbation
No Autosampler interface

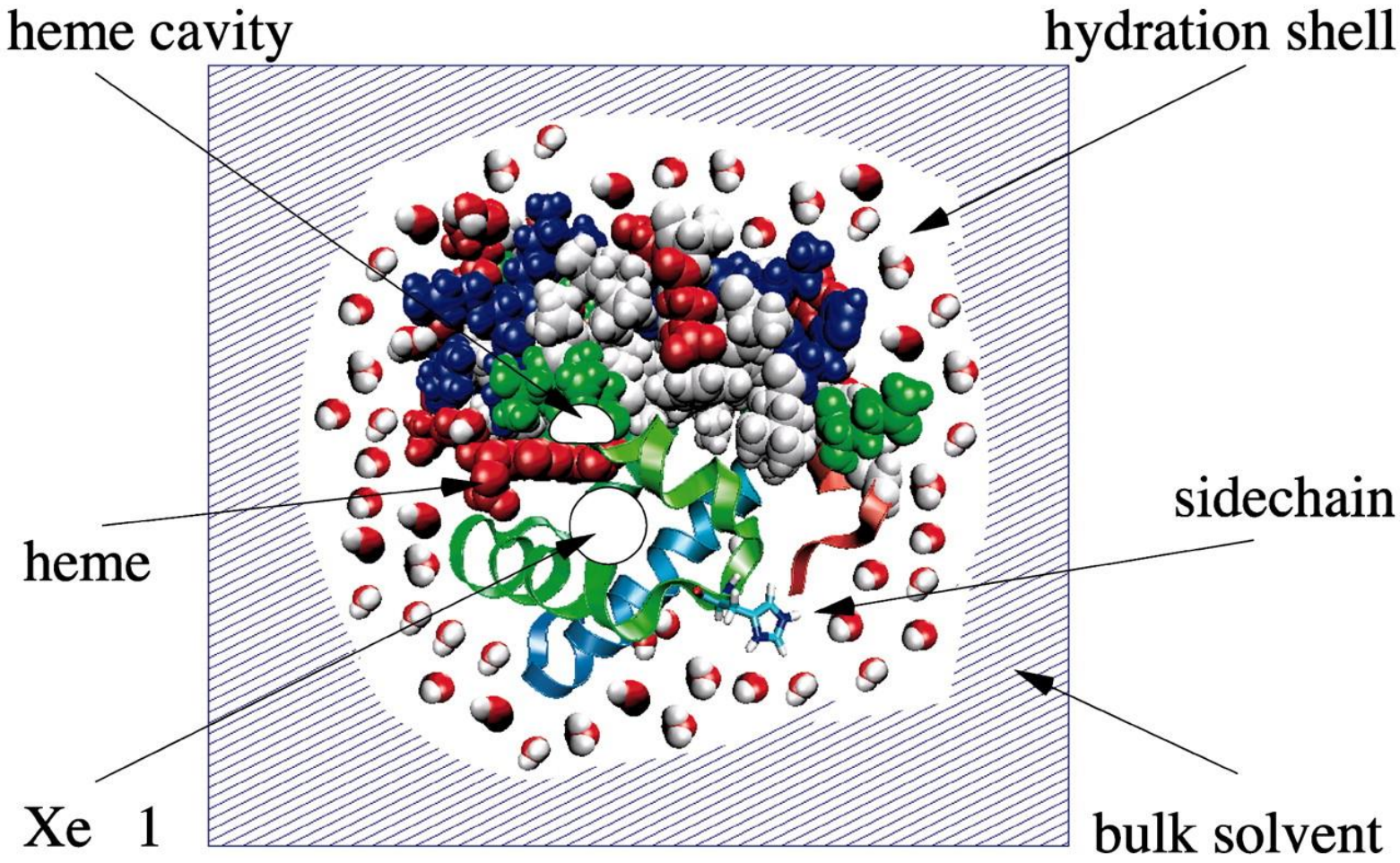
- Pressure perturbation: the heat change in a biopolymer sample caused by a pressure jump.
- Nano-DSC can alter pressure quickly and smoothly, to 6 ATM.
- Heat corresponds to the work done by the pressure to create a volume change.
  - Thermal expansion determined, which is correlated with hydration of the biopolymer.
- Volume change can also be correlated with tightness of packing of protein interior (chymotrypsinogen is more hydrophobic than ribonuclease).



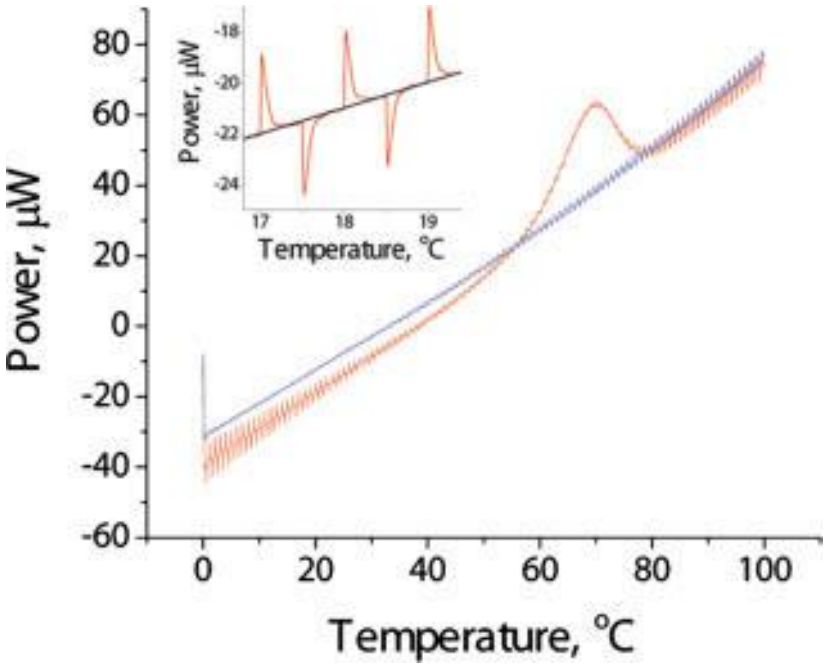


Lin *et al., Anal. Biochem*. **302**, 144-160, 2002

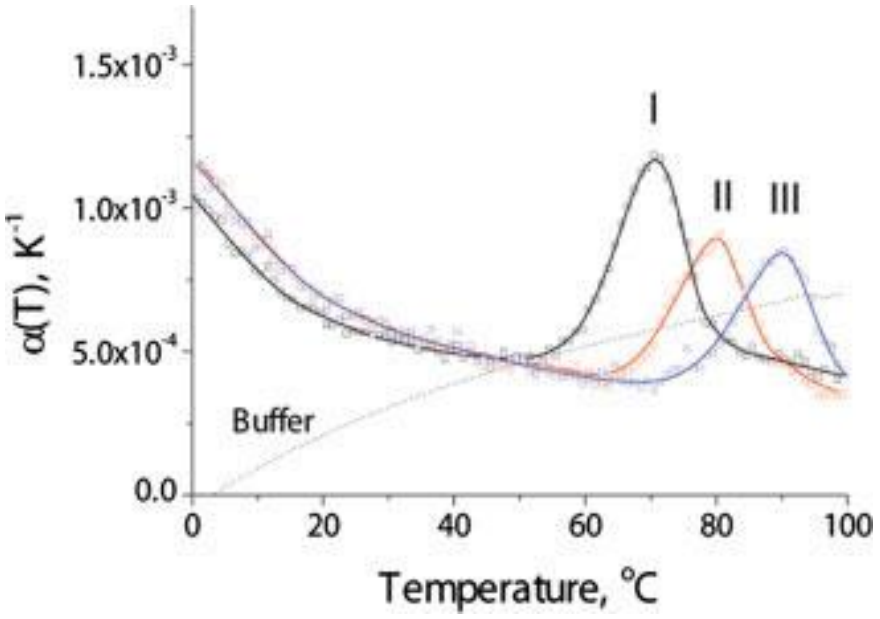
Shell v. Bulk Solvent



Pressure Perturbation



Pressure perturbation scan of 13.4 mg/mL 12bp dsDNA against buffer at 0.1˚ K/min and 1 to 5 Atm pressure pulses.



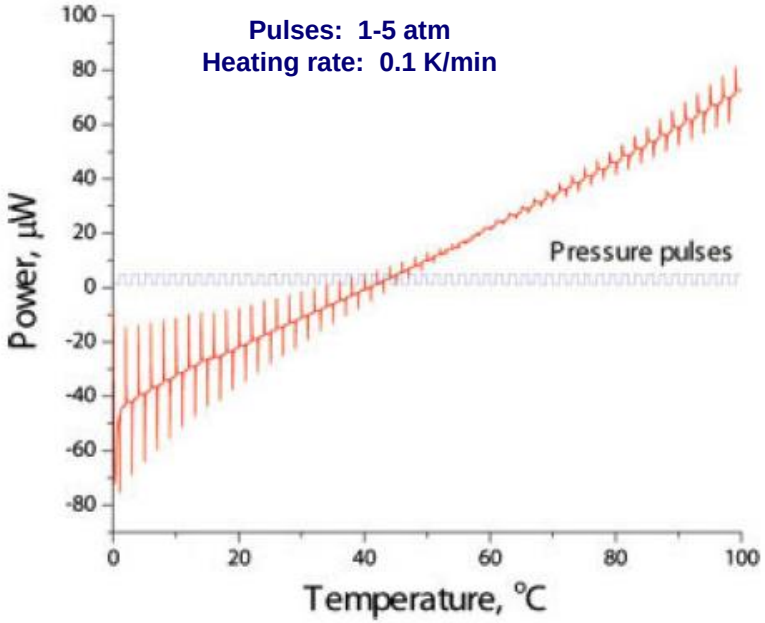
Thermal expansion coefficients of three different 12bp dsDNA

Pressure perturbation experiments provide information on the temperature-induced changes in samples

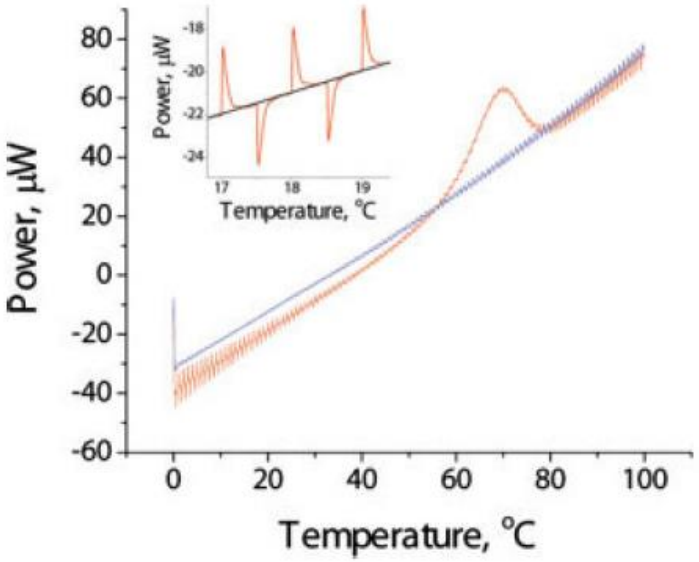
Dragan et al., Biopolymers, 91(1), 2008

Application of pressure perturbation

PPC of Buffer



PPC of 12bp DNA Duplex I

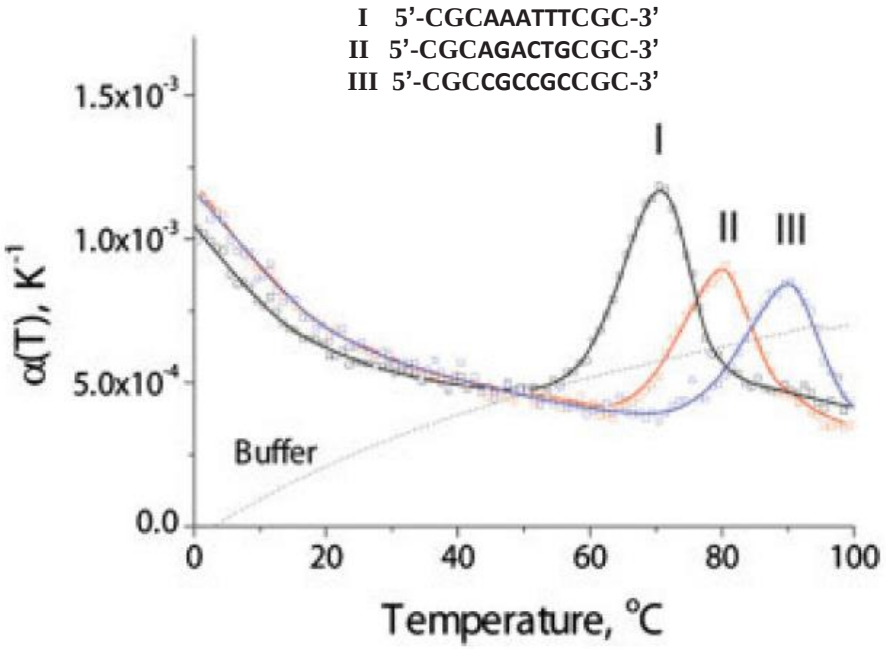


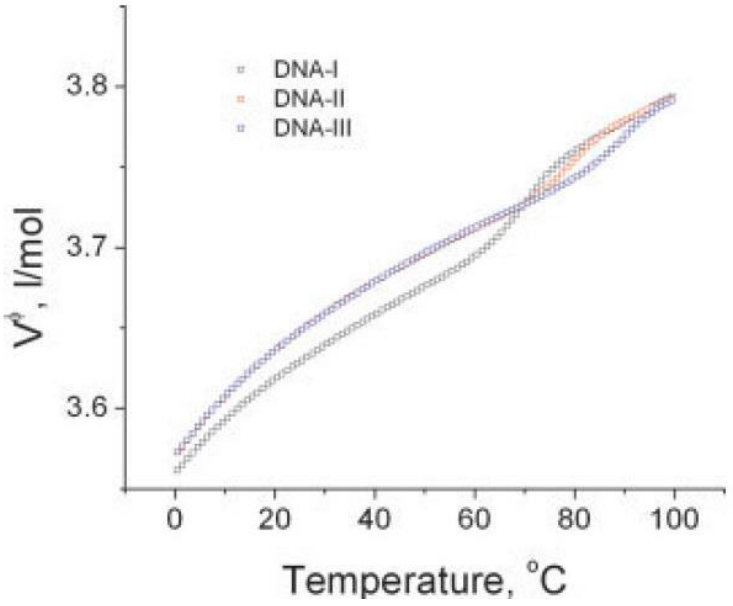
- Pressure perturbation effects are different for the DNA solution and the Buffer and they change in different ways with temperature increase.

Dragan, Russell, Privalov; Biopolymers 91:95-101. 2009

Application of pressure perturbation

PPC of Three Different 12bp DNA Duplexes





- Temperature reduction results in a decrease of the partial volumes of all three considered duplexes
- The decrease of the partial volume on formation of duplex I is about 20 ml/mol greater than that of the two other – Duplex one has contiguous -AAATTT- stretch
- Difference in partial volumes must be attributed to the differences in the volumes of the hydrating water. It follows from the above results that the volume of water hydrating the duplex containing the -AAATTT- stretch is considerably smaller than the volume of water hydrating the two other duplexes

Dragan, Russell, Privalov; Biopolymers 91:95-101. 2009

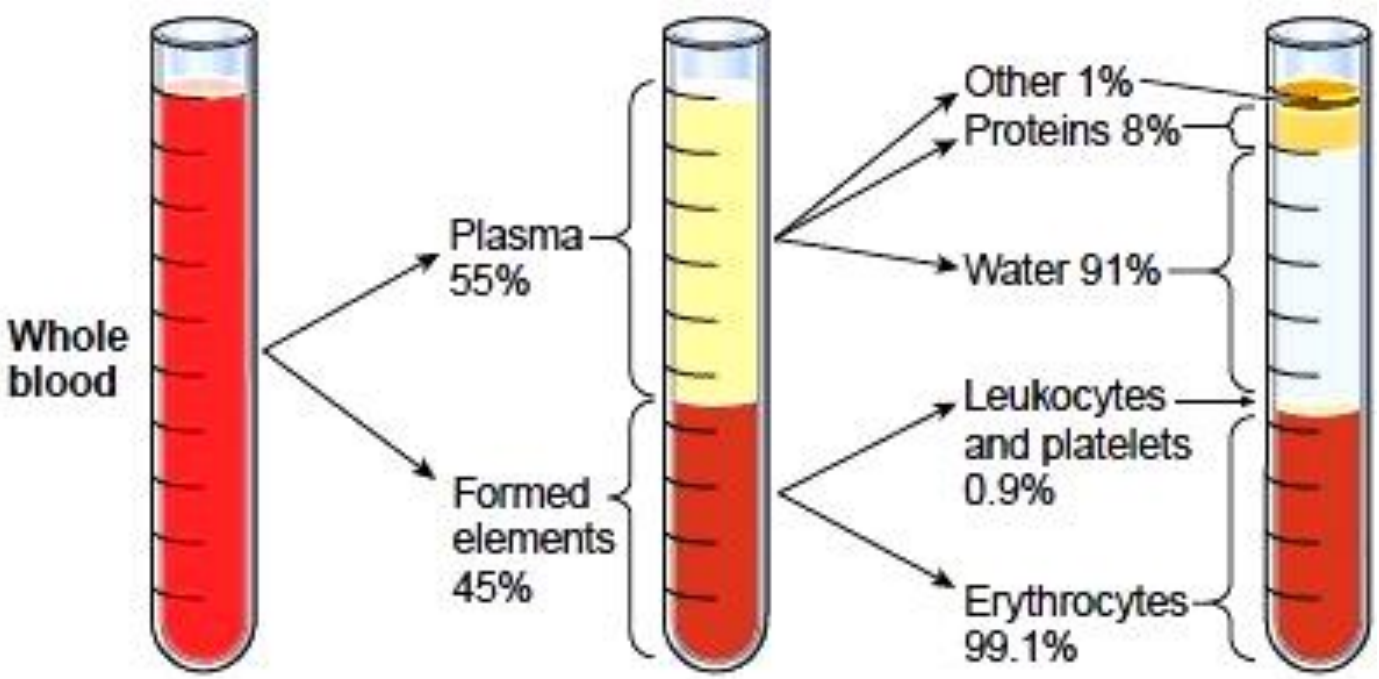
Up and Coming Application

Plasma Proteome - Peptidome

- 3000 individual proteins / peptides
- Biomarkers for diagnostic purpose
- Therapeutic monitoring
- Minimally invasive and safe access

- Peptides related to diseases expected to increase
- Increased binding / interaction with huge proteins found

Whole blood fractions

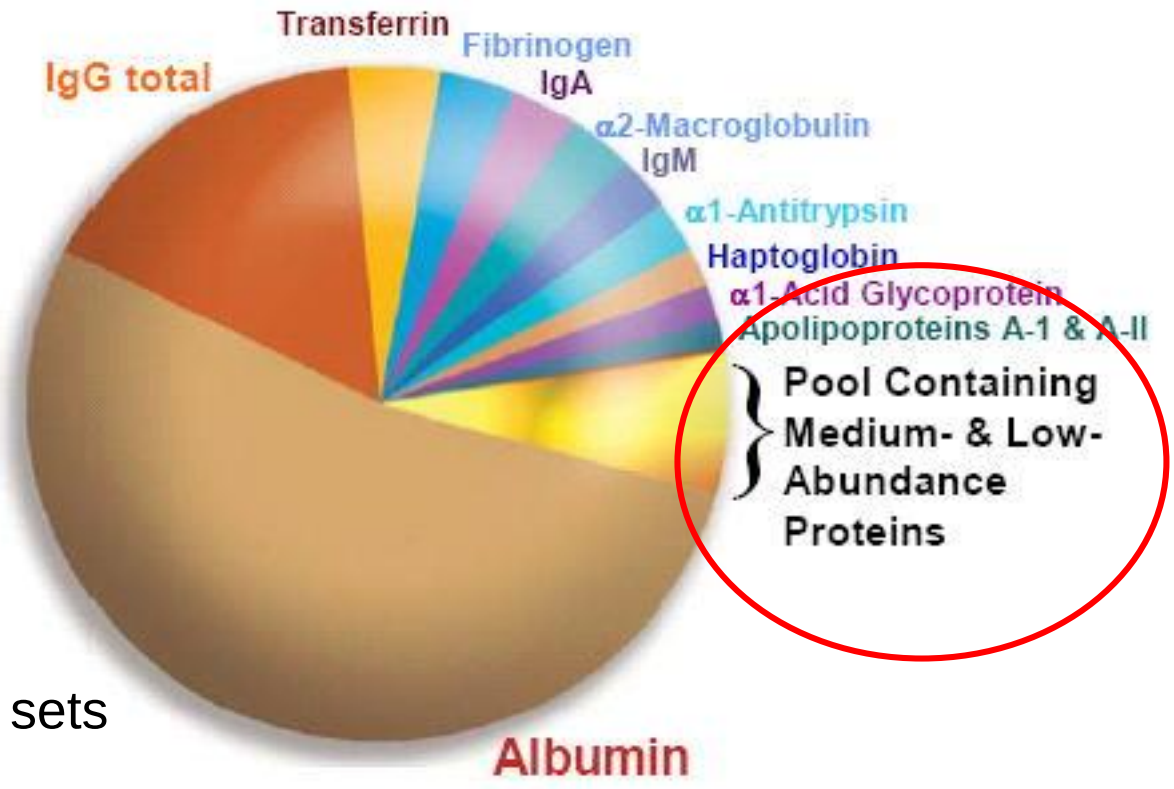


picture from: www.encyclopedia.lubopitko-bg.com Corporation

Major Plasma Proteins

- 10 proteins make up 90% of the mass of plasma proteome
- next 10-12 proteins account for another 9% (2D GE, MS)

1% remains (LAP)
- biomarkers
- peptides



Disease related:
- no single peptides, rather sets
- changed composition
- peptides complexed to HSA or Ig

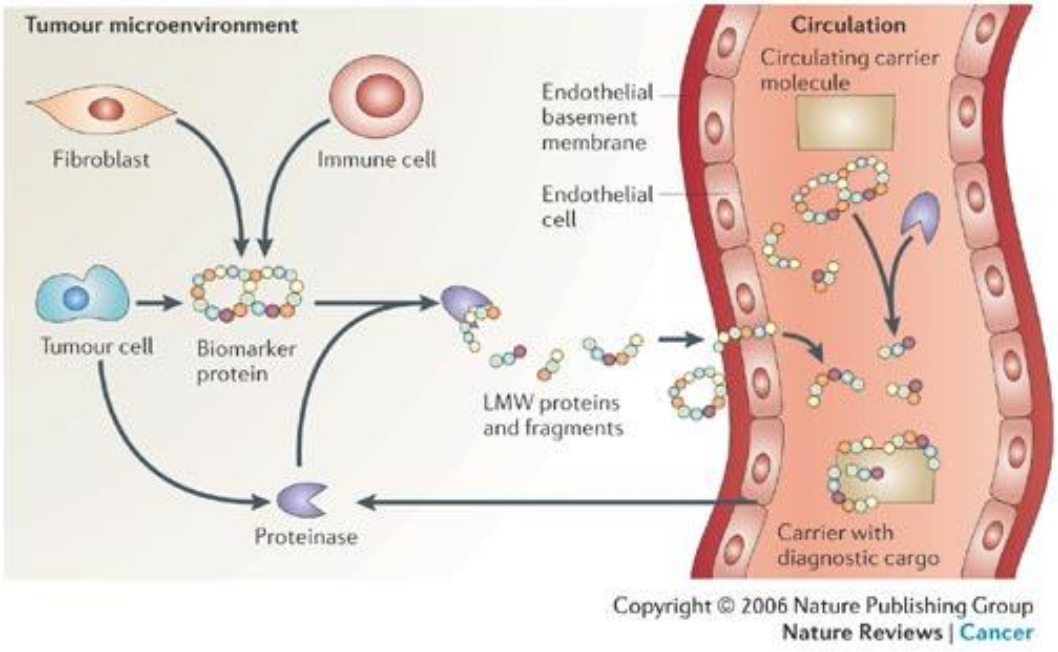
picture from: pssbio.com

Lookout for cancer / disease biomarkers

inside plasma huge proteins:
albumin
globulines
Fibrinogen…

biomarker / peptides rather small

small changes in size and charge

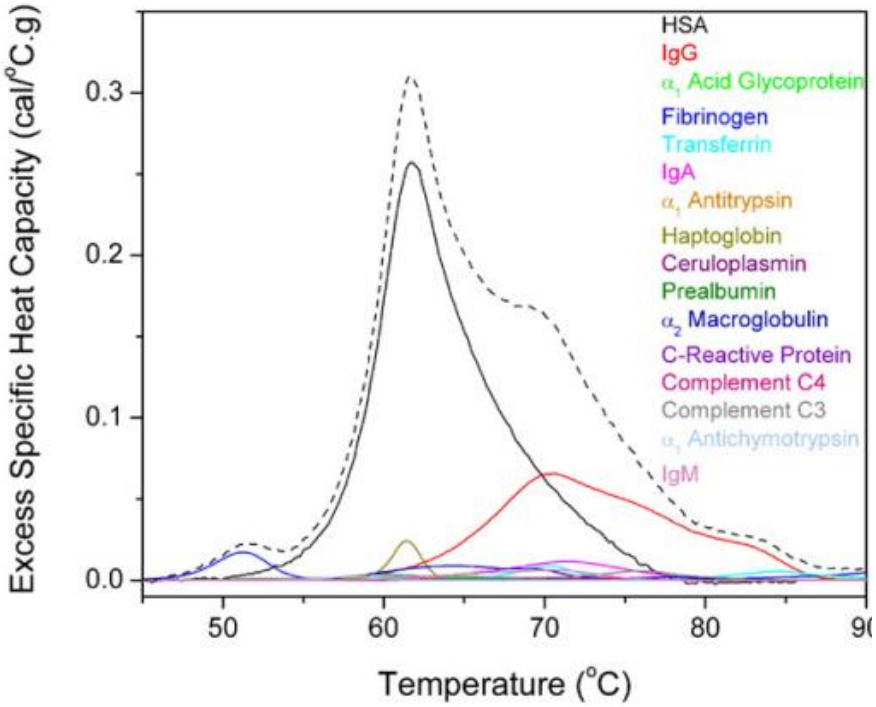


easy to miss in
electrophoresis
mass spectrometry

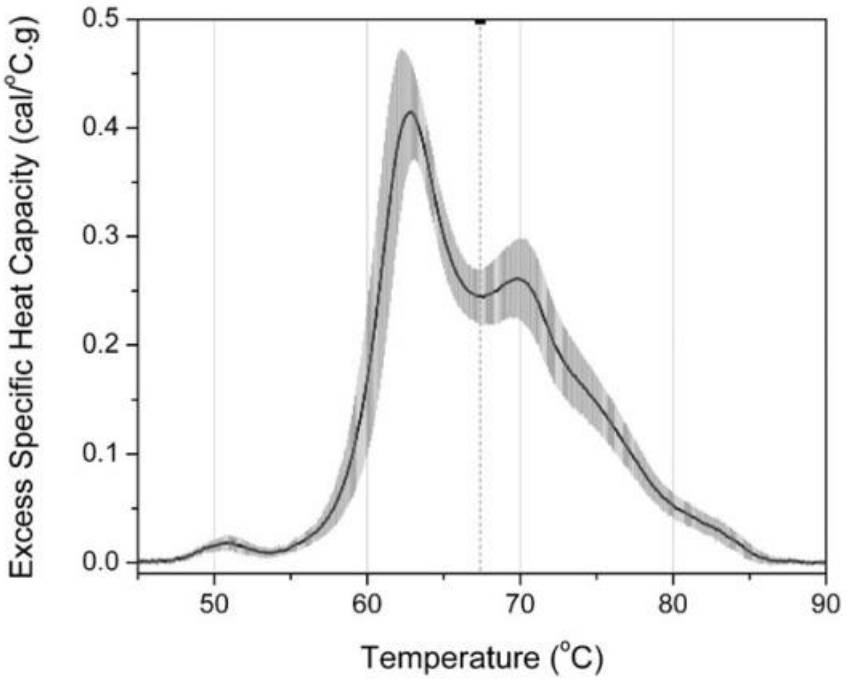
DSC is sensitive to
composition and interaction

Characterization of plasma proteome from healthy controls

Thermograms of the 16 most abundant plasma proteins



Plasma of healthy individuals (9♂ and 5♀)
x ± sd



Garbett et al. (2009), Experimental and Molecular Pathology 86:186-191

Setup for plasma in DSC measurements

Simple procedure

1. Sample preparation (example from Garbett et al.)

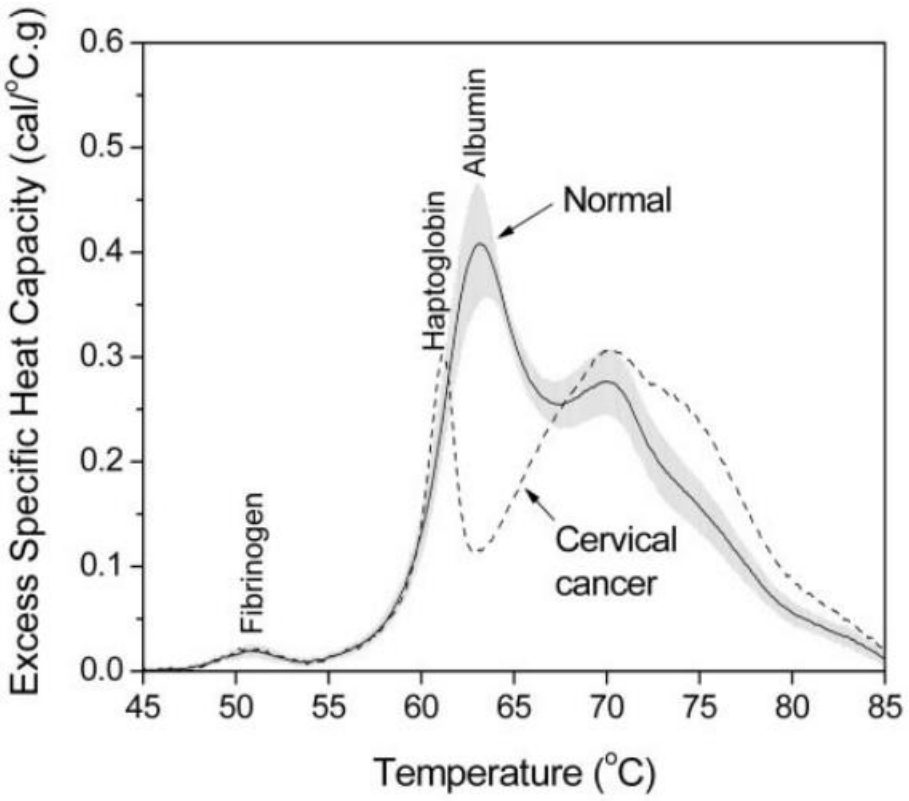
Filtration 45µm cellulose acetate
Concentration 25 fold dilution
buffer PBS pH 7.5 (10mM PO4, 150mM NaCl, 3.8g/L NaCitrat)

2. Experimental procedure

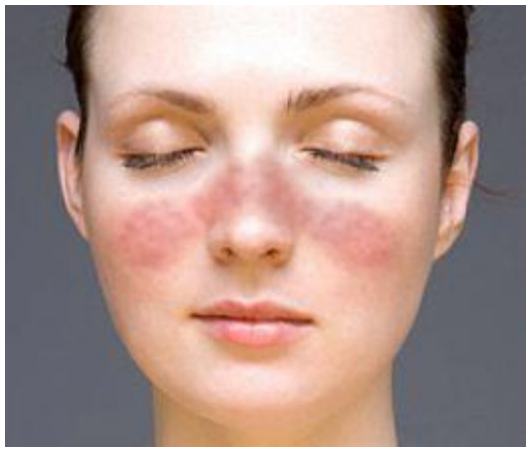
scan rate	1° C / min
scan range	10 – 90° C
baseline correction	buffer / buffer blank

Systemic lupus erythemathosus (SLE) and Cervical Cancer

- Thermograms of both diseases show distinct Haptoglobin peak due to Albumin stabilization (T_m shift), no significant change in albumin conc. (proved by electrophoresis)

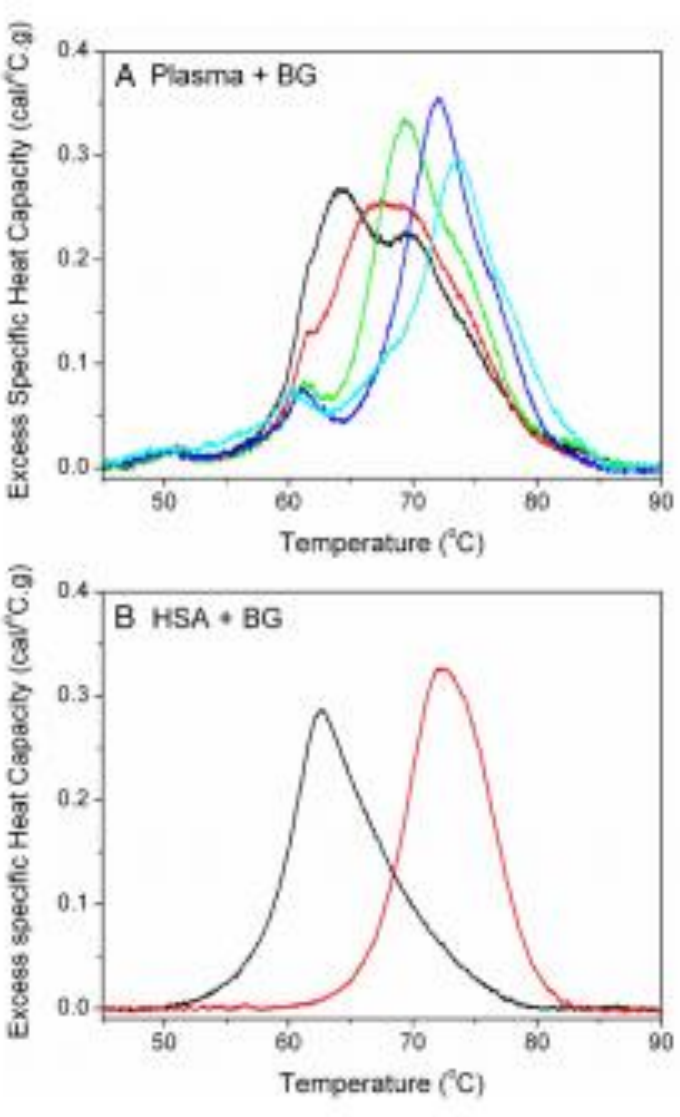


- Haptoglobin peak also found in 'lupus' autoimmune disease:typical 'butterfly rash'



simeyc.hubpages.com

Shifts in thermograms due to binding on albumin



Proof of concept:
Bromocresol Green (BG) mixed to Plasma (A) and Albumin (B):

- T_m shifts to higher stability
- higher [BG], higher Tm shift
- resemblence to disease thermograms
- reveals lower abundance proteins

Interactomics!

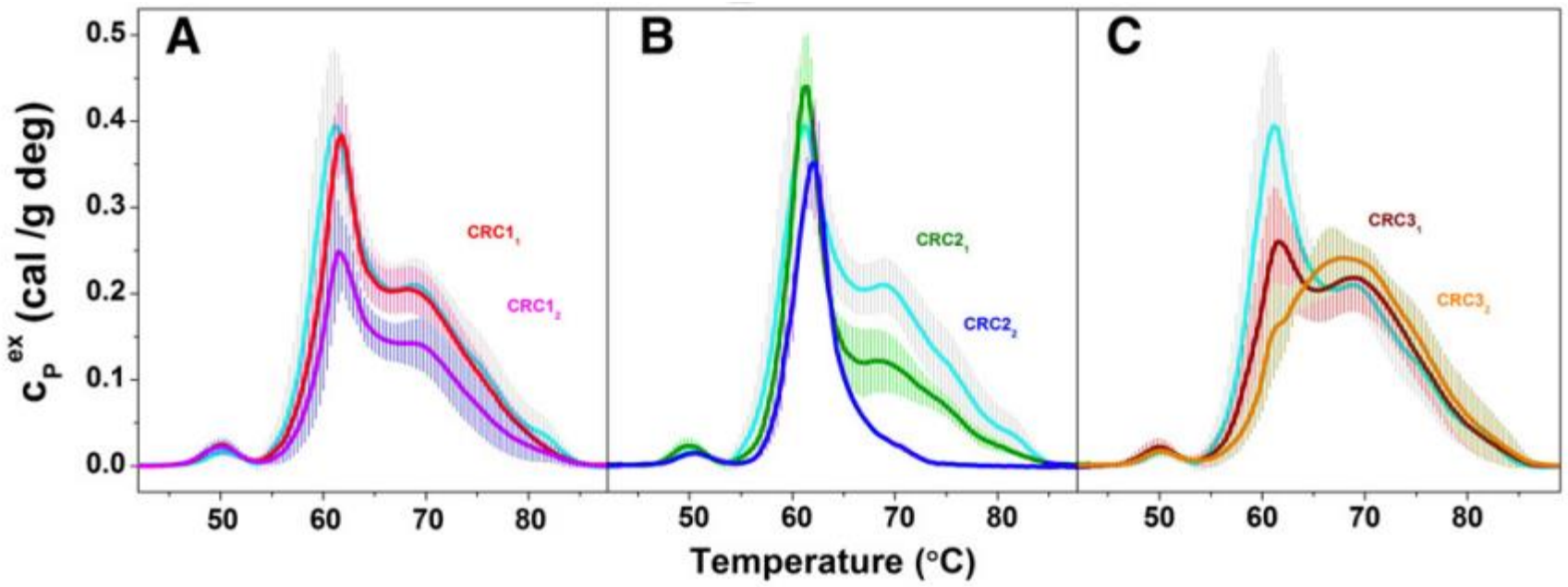
Garbett et al. (2009), Exp and Mol Pathology 86:186-191

Colorectal Cancer (CRC)

healthy control (32 volunteers)

CRC1, CRC2, CRC3 classification

- Cp-ratio T2/T3 comparison between healthy and cancerous sample
- 0.8 K/min, 20-95˚C, buffer blank

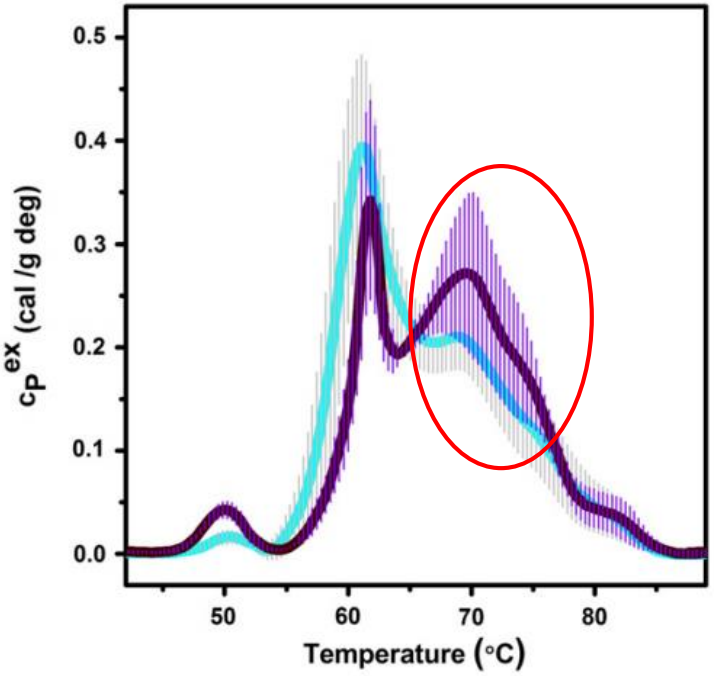


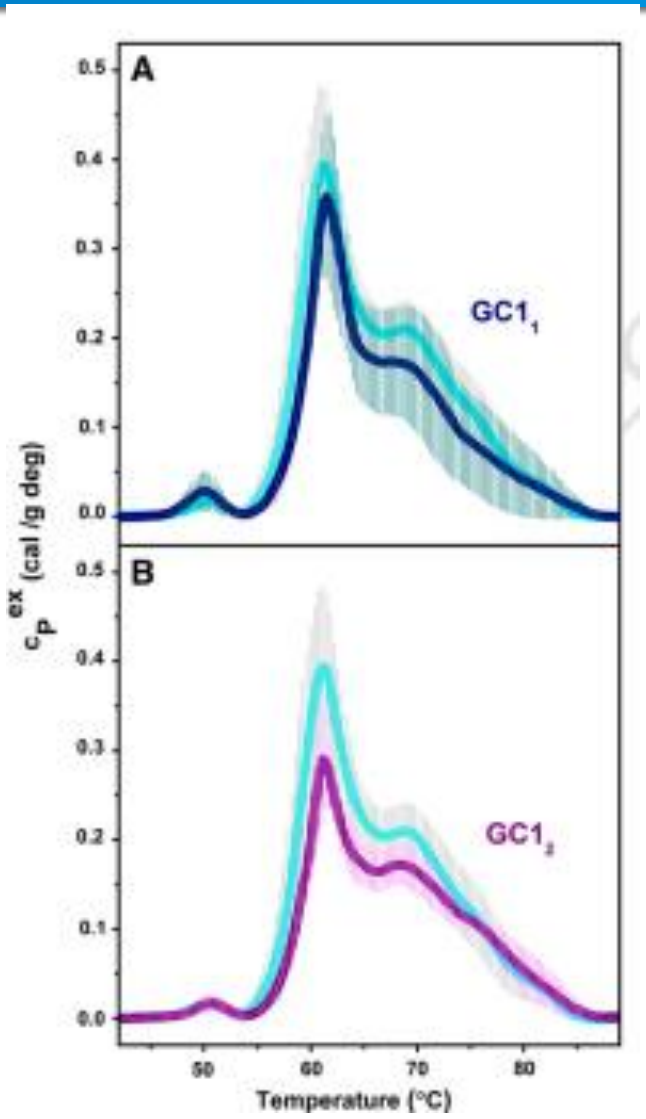
Todinova et al. (2012), Biophimica et Biophysica Acta
http://dx.doi.org/10.1016/j.bbagen.2012.08.001

Non cancerous soft tissue inflammation vs Gastric cancer

Inflammation

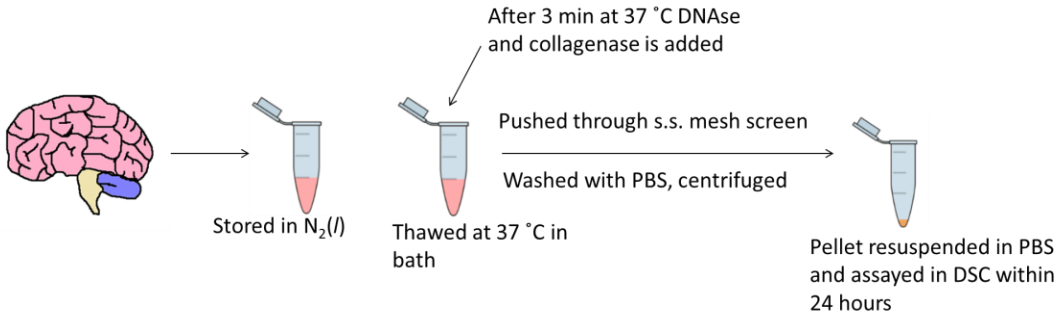
- increasing globulins
- decreasing albumins

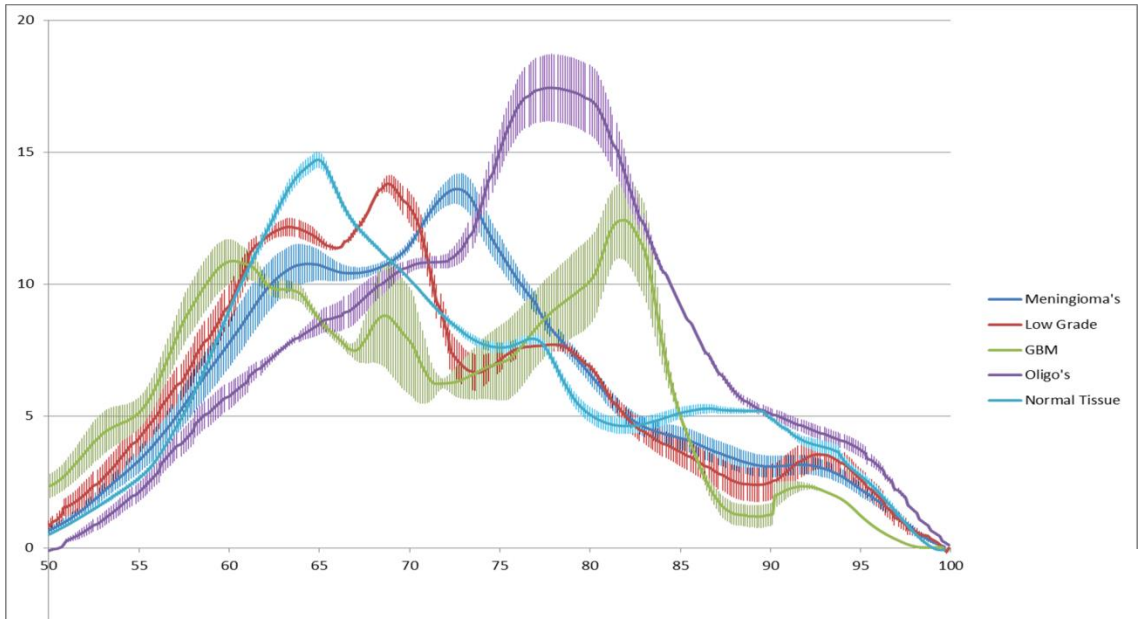


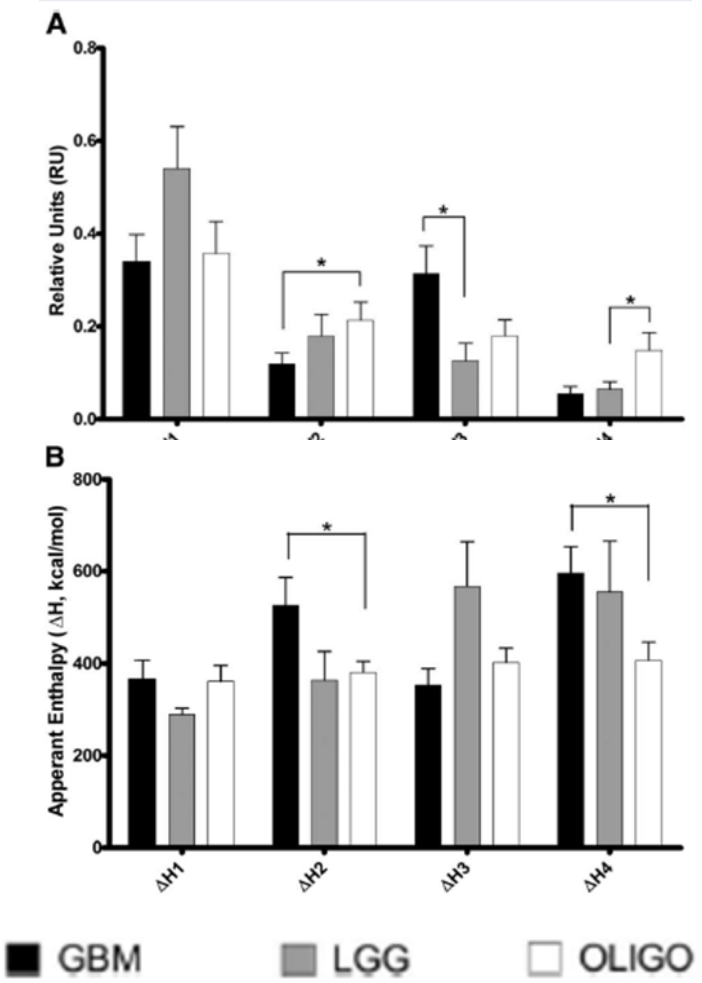


Todinova et al. (2012), Biophimica et Biophysica Acta
http://dx.doi.org/10.1016/j.bbagen.2012.08.001

Brain Tumor Study







Chagovetz A, Quinn C, Demarse N, Hansen L, Chagovetz A, Jensen, R. Differential Scanning Calorimetry of Gliomas: A New Tool in Brain Cancer Diagnostics? *Neurosurgery* 2013 73(2)289-295

- Only T_{m4} showed statistical differences between types.

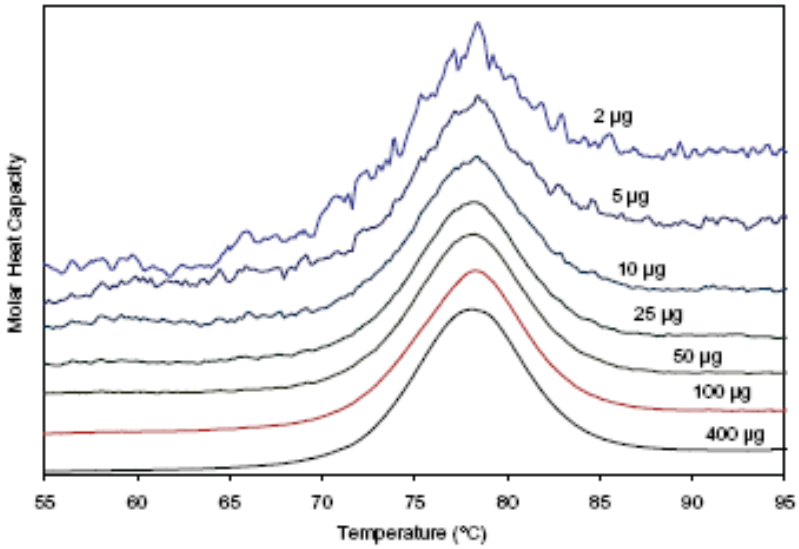
DSC Utility

- Consider the systems that our users put into the **Nano DSC**: blood serum, brain tissue, cerebral spinal fluid, liposomes, micelles. We don't limit ourselves to single protein unfolding or even only proteins!

- DSC can quantify the cooperativity of the unfolding. The relationship of ΔH_{cal} and ΔH_{VH} provide interesting information regarding the cooperativity of unfolding as well as the oligiomeric state of the protein.

Experimental: Getting Started

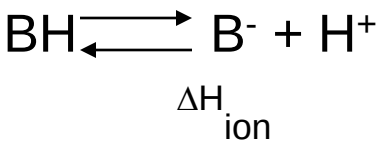
How much protein is required for a DSC scan?



Lysozyme in cell (µg)	Calorimetric		van't Hoff	
	ΔH (kJ mol^{-1})	ΔS (kJ K^{-1} mol^{-1})	T_m (°C)	ΔH (kJ mol^{-1})
400	512	1.46	78.0	515
100	512	1.46	78.0	509
50	517	1.47	77.9	513
25	513	1.46	77.8	513
10	515	1.47	78.0	515
5	490	1.40	78.1	510
2	503	1.43	77.8	499

Choice of Buffer

- All buffers have an ionization enthalpy:



- Buffers with high ionization enthalpy undergo large change in pH as temperature increases (e.g. TRIS)
- Use buffers with low ionization enthalpy: acetate, formate, citrate, glycine
  - Quaternary amine buffers have high ionization enthalpies and should be avoided
- Use only thermostable additives.
  - For reducing agents, TCEP is preferable to β-mercaptoethanol or DTT.
- Caution: viscous additives, especially preservatives and stabilizers such as glycercol and detergents, can trap air bubbles, and dialyze very slowly.

Buffer Thermal Stability

Temp (°C)	acetate	MES	PIPES	Phos	BES	MOPS	TES	HEPES	Tris	Bicine	TAPS	CAPS
0	4.73	6.35	6.98	6.92	7.40	7.44	7.93	7.73	8.98	8.63	8.97	11.06
10	4.71	6.24	6.90	6.86	7.23	7.30	7.71	7.58	8.65	8.45	8.69	10.74
20	4.70	6.15	6.84	6.82	7.08	7.16	7.50	7.46	8.34	8.28	8.42	10.43
30	4.70	6.06	6.77	6.79	6.93	7.04	7.31	7.34	8.06	8.12	8.18	10.15
40	4.70	5.97	6.70	6.77	6.79	6.91	7.13	7.22	7.81	7.97	7.95	9.88
50	4.71	5.89	6.64	6.76	6.86	6.80	6.98	7.11	7.57	7.83	7.74	9.63
60	4.73	5.81	6.58	6.78	6.54	6.69	6.81	7.00	7.35	7.70	7.54	9.39
70	4.75	5.74	6.53	6.77	6.42	6.58	6.66	6.89	7.15	7.58	7.38	9.16
80	4.78	5.67	6.47	6.79	6.31	6.48	6.53	6.79	6.96	7.46	7.18	8.94
90	4.81	5.60	6.42	6.82	6.21	6.38	6.40	6.69	6.76	7.35	7.02	8.74
100	4.84	5.53	6.37	6.85	6.11	6.28	6.29	6.60	6.62	7.25	6.87	8.84

(Phos: phosphate. Values from H. Fukada and K. Takahashi (1987), Laboratory of Biophysical Chemistry, College of Agriculture, Sakai, Osaka, Japan.)

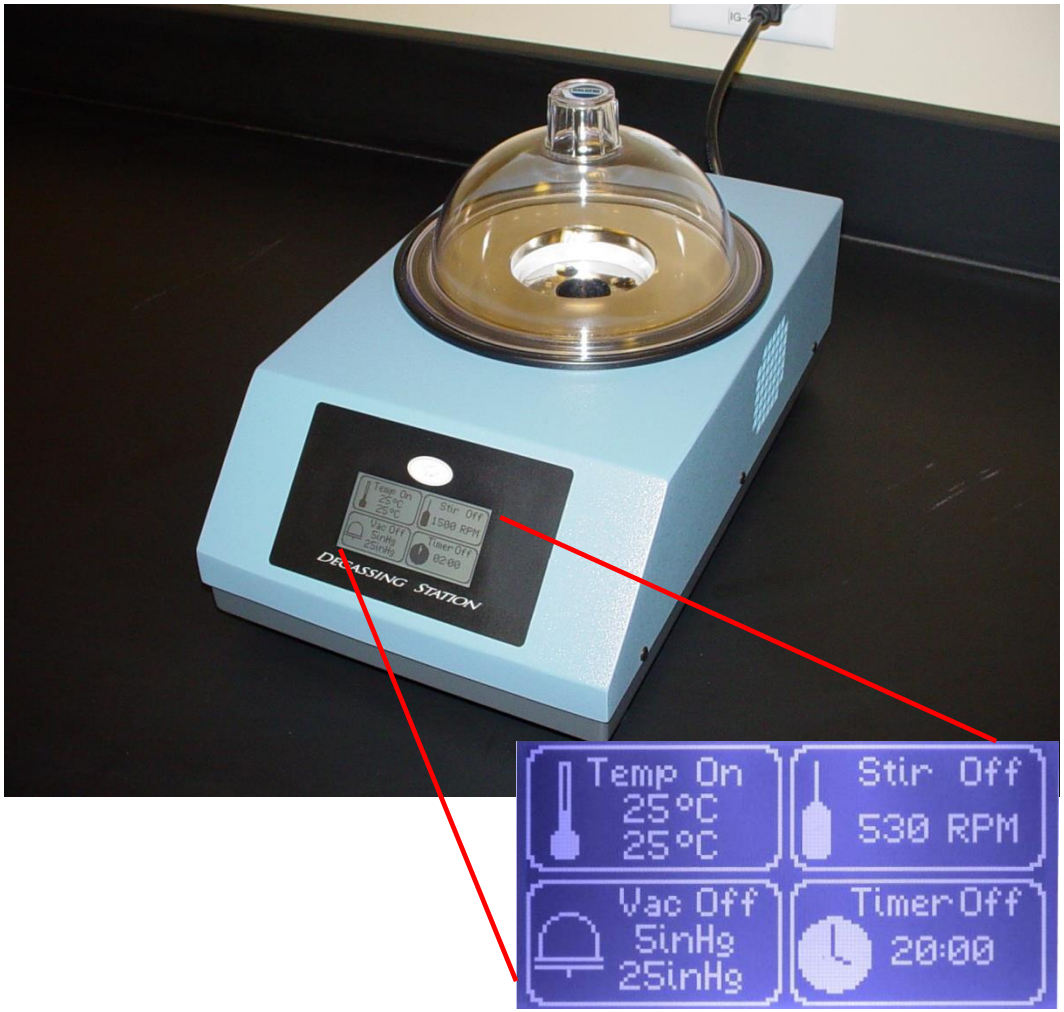
Sample scans

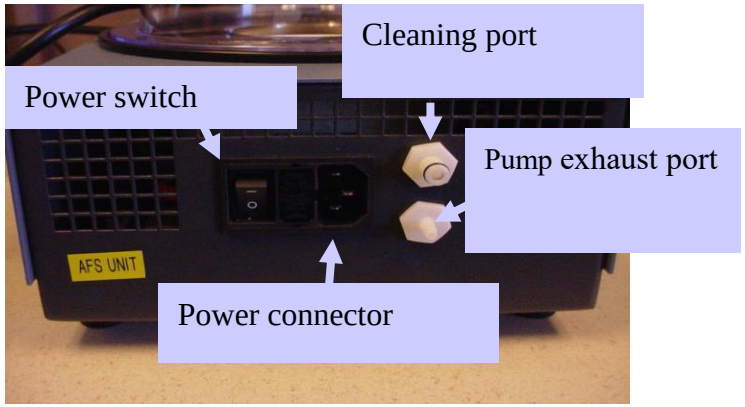
- Accurate concentration determination is critical for accurate ΔH, ΔC_p. Typical concentration range is 0.1 – 1 mg/mL,
- Sample volume of ~0.55 mL is sufficient to overfill the 0.3 mL cell
- For added reproducibility, load 'on the fly':
  - Release pressure at ~ 30 ^oC on first cooling scan, remove pressure handle
  - Load degassed sample while down scan continues
  - Sample will be scanned on next heating cycle
- Repeat to establish reproducibility of sample thermogram
- Rescan same sample (reversibility). Vary sample concentration (oligomerization), scan rate (kinetic effects).
- Hint: Use fast (2 ^oC/min) scan rate for very dilute samples, slow (0.5 ^oC/min) for complex (multi-domain, multi-subunit) samples
  - Note: Conditioning and baseline repeatability scans are only necessary when changing buffer composition.

Sample Dialysis and Degassing

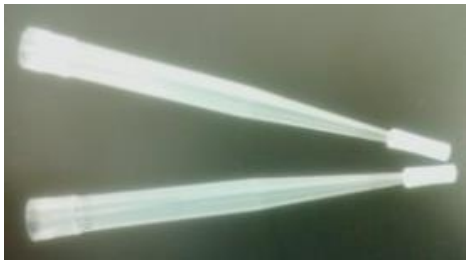
- DSC measures the small heat capacity differences between buffer in reference and sample sides. It is critical to match buffer composition in both cells. It is suggested to dialyze sample extensively to remove low molecular weight contaminants.
- Increased temperature results in decreased solubility of dissolved gases, causing bubble formation. Degas sample and dialysis buffer (for reference cell) under vacuum for 5-15 min at about 0.3 atmospheres.
- Bubble formation and boiling are retarded by pressurizing sample and reference cell to 3-6 atmospheres during scanning experiment.
- Ensure cells are scrupulously clean and do not introduce air bubbles, which can affect noise and repeatability of the scans
  - e.g. 1 μL air bubble can cause a 70 μW offset at 1 ^{o}C/min

Degassing Station





Manual Load - Capillary



- Use pipet tips with small piece of silicon tubing to manually load cells
- Insert the the silicon tubing on the sample or reference cell and fill by rocking the solution
- Cap one side of the sample and reference side with a black vinyl cap

- From the Nano DSC Getting Started Guide. Available on any computer with DSC Run installed.

Nano DSC Data Analysis

- Flexible DSC data fitting models
  - Two State
  - Two State Scaled
  - General
  - Gaussian
- Flexible Overlay Graphing
  - Flexible display of multiple graphs
  - User selectable format for graph export
- Statistics on results (T_m, ΔH)
  - User adjustable fitting iterations
  - Confidence interval calculations
  - Visual graph of fitting iteration results
  - Statistics on fit parameters (T_m, ΔH)
- Batch Processing
  - Tabular listings
  - Easy analysis of multiple files

Nano DSC Data Analysis - Models

- Three models available in NanoAnalyze
  - Two-State
  - Two-State Scaled
  - General
- Modeling of data using “psuedo” van’t Hoff method

This ‘psuedo’ van’t Hoff expression does not depend on the absolute values of ΔC_p and ΔH and consequently neither on the possible errors in determining the concentration of the studied material nor on the calibration of the scanning calorimeter.

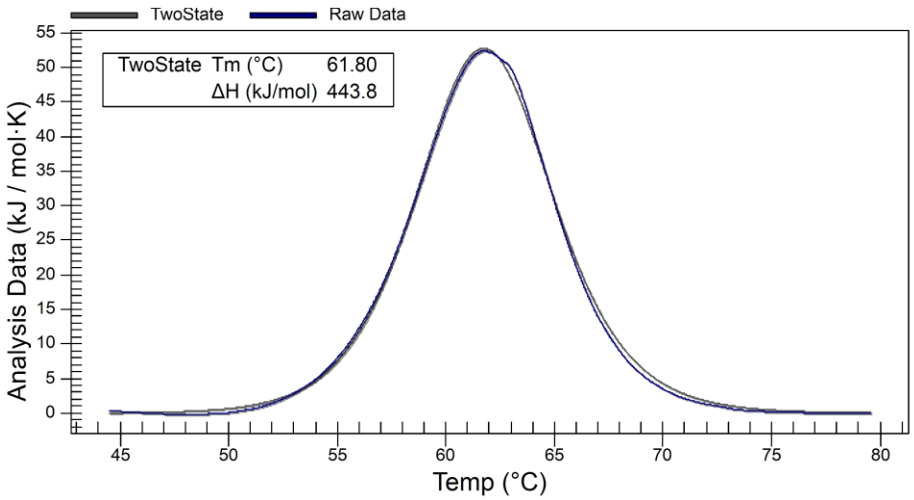
P.L. Privalov and S.A. Potekhin “Scanning Microcalorimetry in Studying Temperature-Induced Changes in Proteins” Methods in Enzymology (1986), v**141**, pages 4-51

Model Definitions: Two-State

- This model assumes the protein to be either folded or unfolded (i.e. two states), and essentially attempts to fit a Gaussian distribution to these states.

- It also assumes that any concentration and MW information entered in the molar heat capacity conversion dialog to be accurate.

- If either of these quantities is off, then the modeled peak will not be able to simultaneously match both the width and height of the data peak.



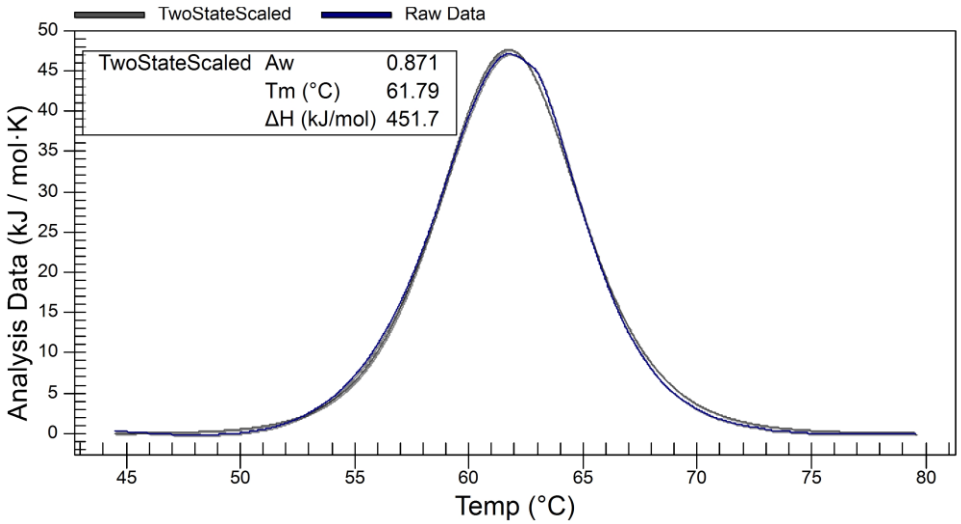
Model Definitions: Two-State Scaled

- The two-state scaled model adds the additional A_w variable, a scaling factor to compensate for errors in the assigned concentration.

- Such errors may arise from not knowing how much of the material in the original solution is in its native folded state and how much may have already denatured.

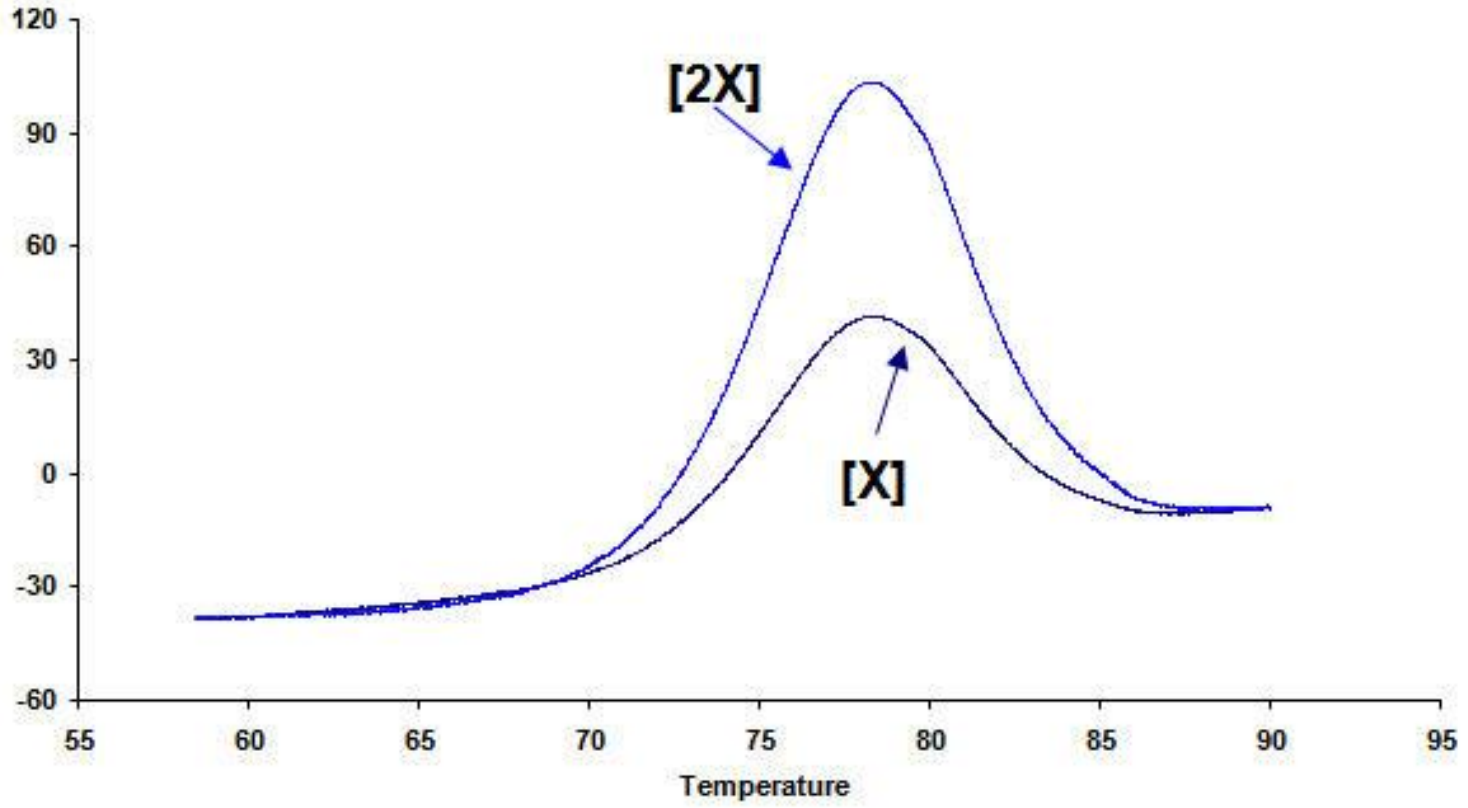
- Use of multiple two-state scaled models is a convenient method for peak deconvolution.

- When using either the simple or scaled two-state model, the data must be baseline-subtracted.



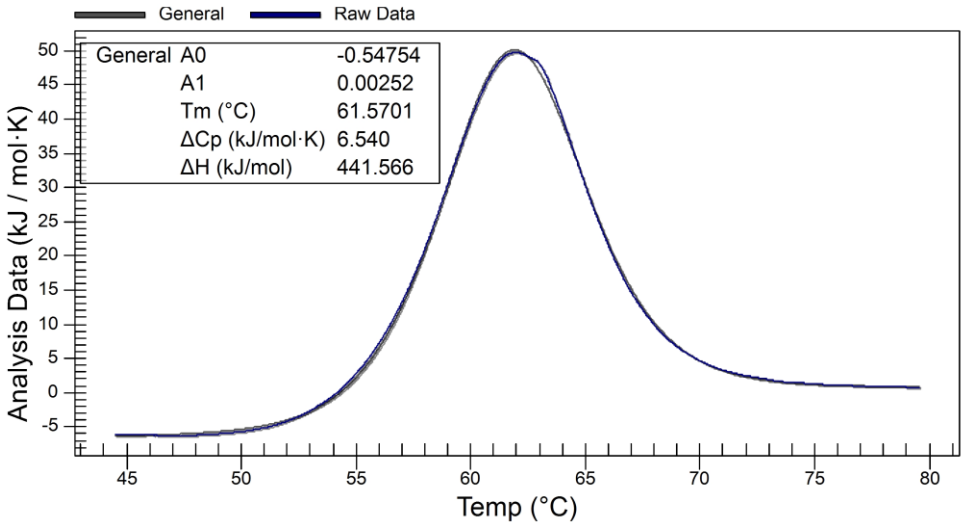
Importance of concentration determination

- ΔH_{cal} is dependent on concentration of the folded protein in sample
- Denatured protein and contaminants will provide incorrect ΔH_{cal}, ΔS, ΔG

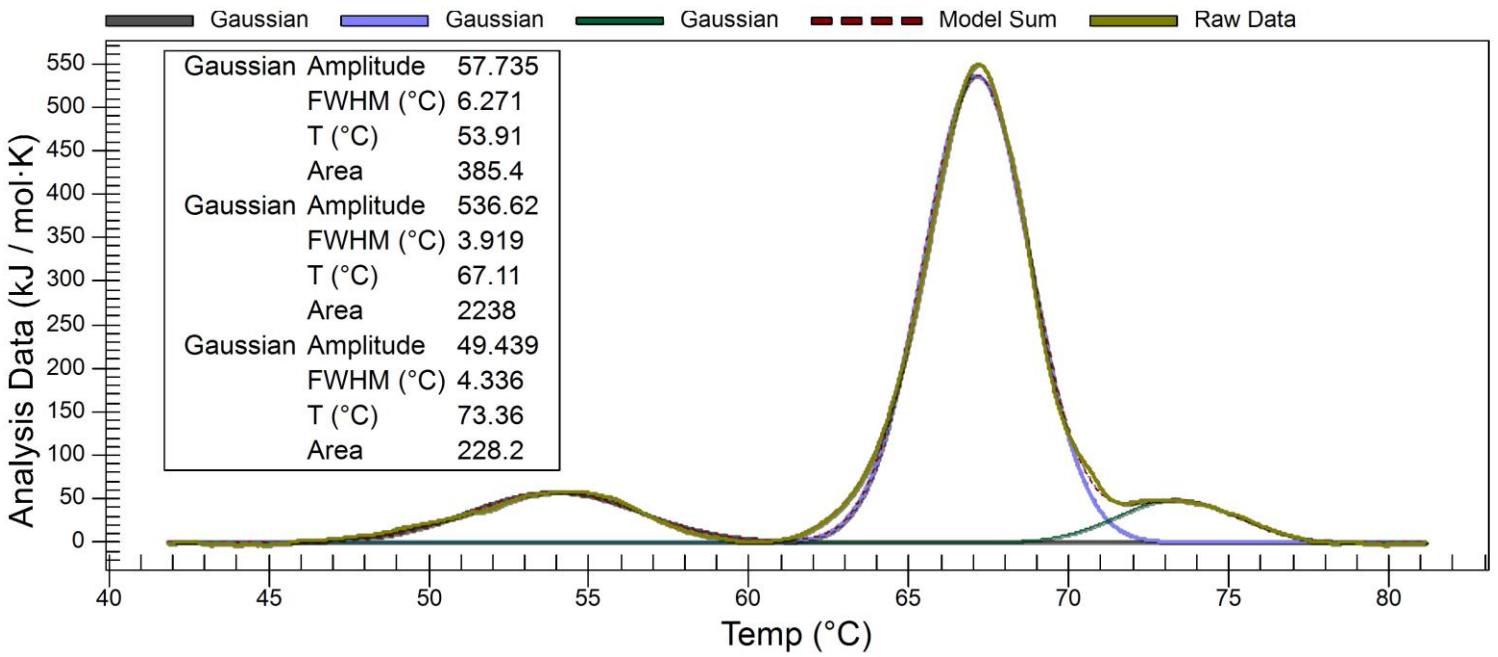


Model Definitions: General

- Most Complex in terms of variables to fit
- This model is typically only used in cases where the user is interested in the ΔC_p before and after the peak, and wants to derive it from a model.
- The A_0 and A_1 variables of this model represent the intercept and slope of the leading baseline (before the transition), respectively.
- Don't subtract the baseline when fitting this model.



Model Definitions: Gaussian



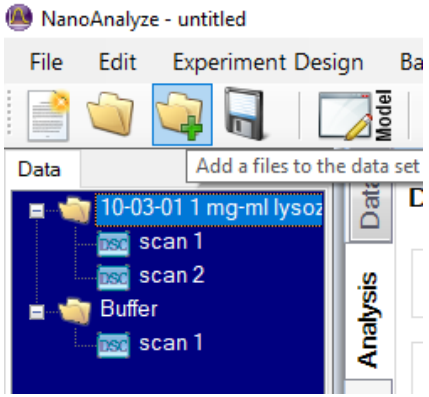
This generic model mathematically describes the symmetric unfolding of a molecule.

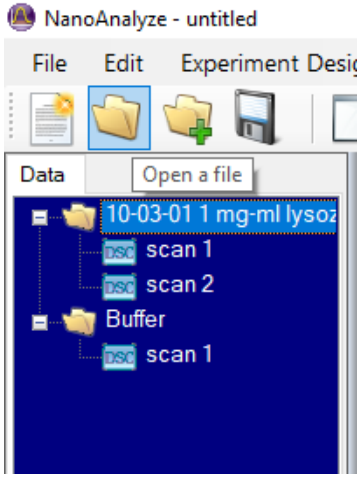
Nano DSC data analysis

Basic steps of DSC data analysis:

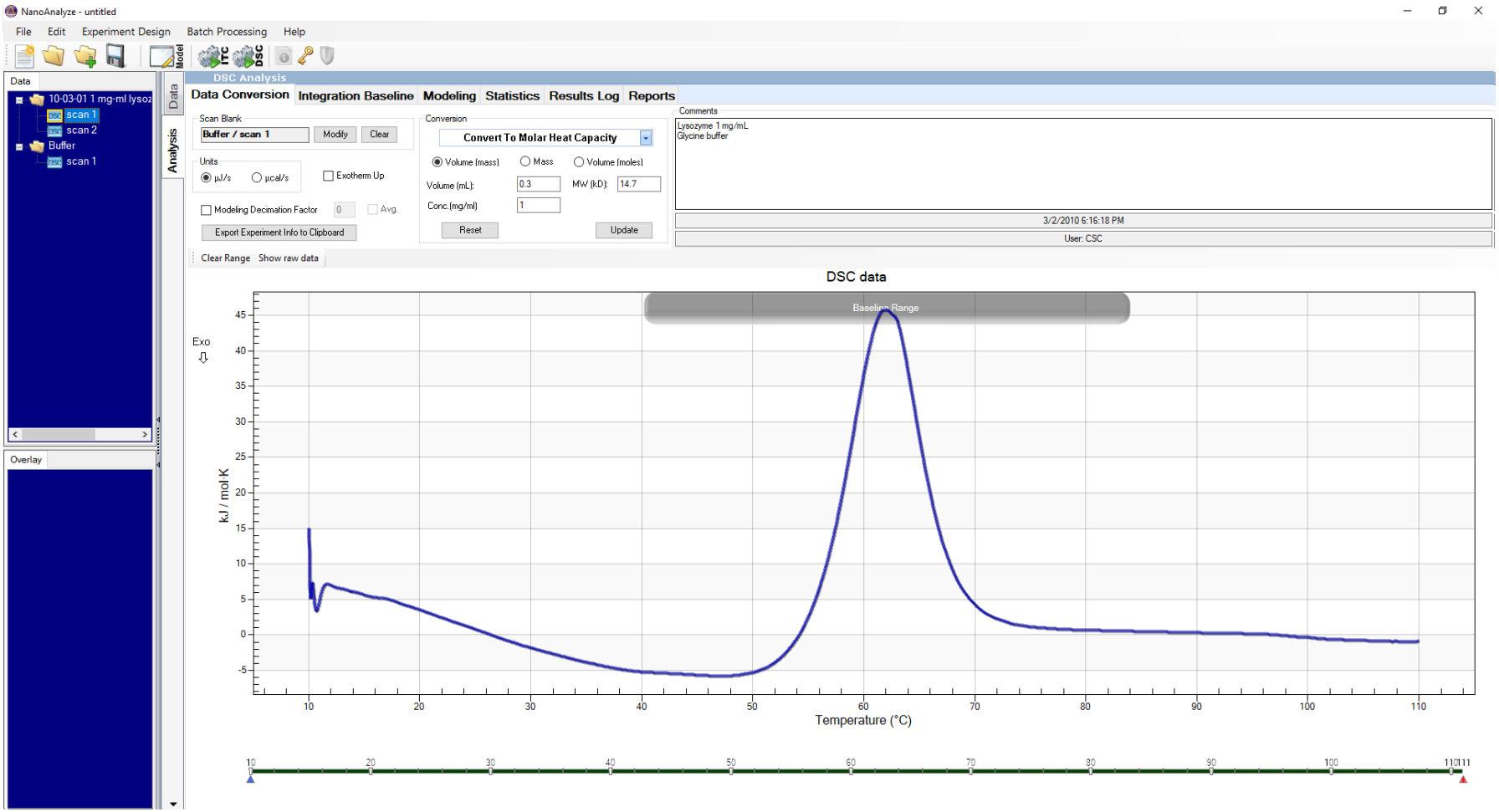
- Add file(s) to NanoAnalyze worksheet
- Chemical baseline subtraction
- Molar heat capacity (MHC) conversion
- Select region of interest
- Apply integration baseline
- Modeling of data

- DSC file types for NanoAnalyze:
  - Raw data file extensions -.dsc files
    - Use Add file
  - Analyzed data in saved sessions - .csc
    - Use Open file

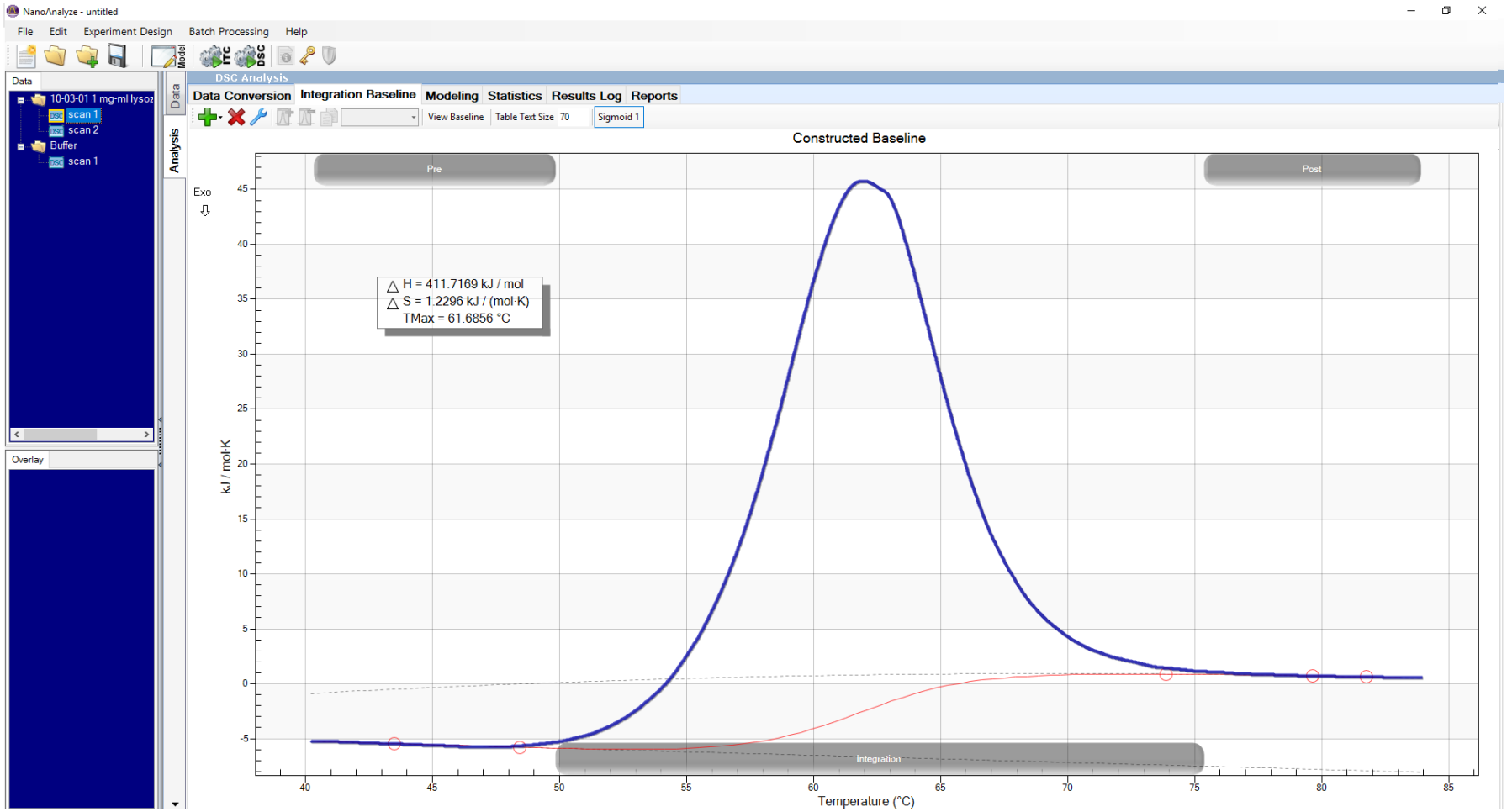




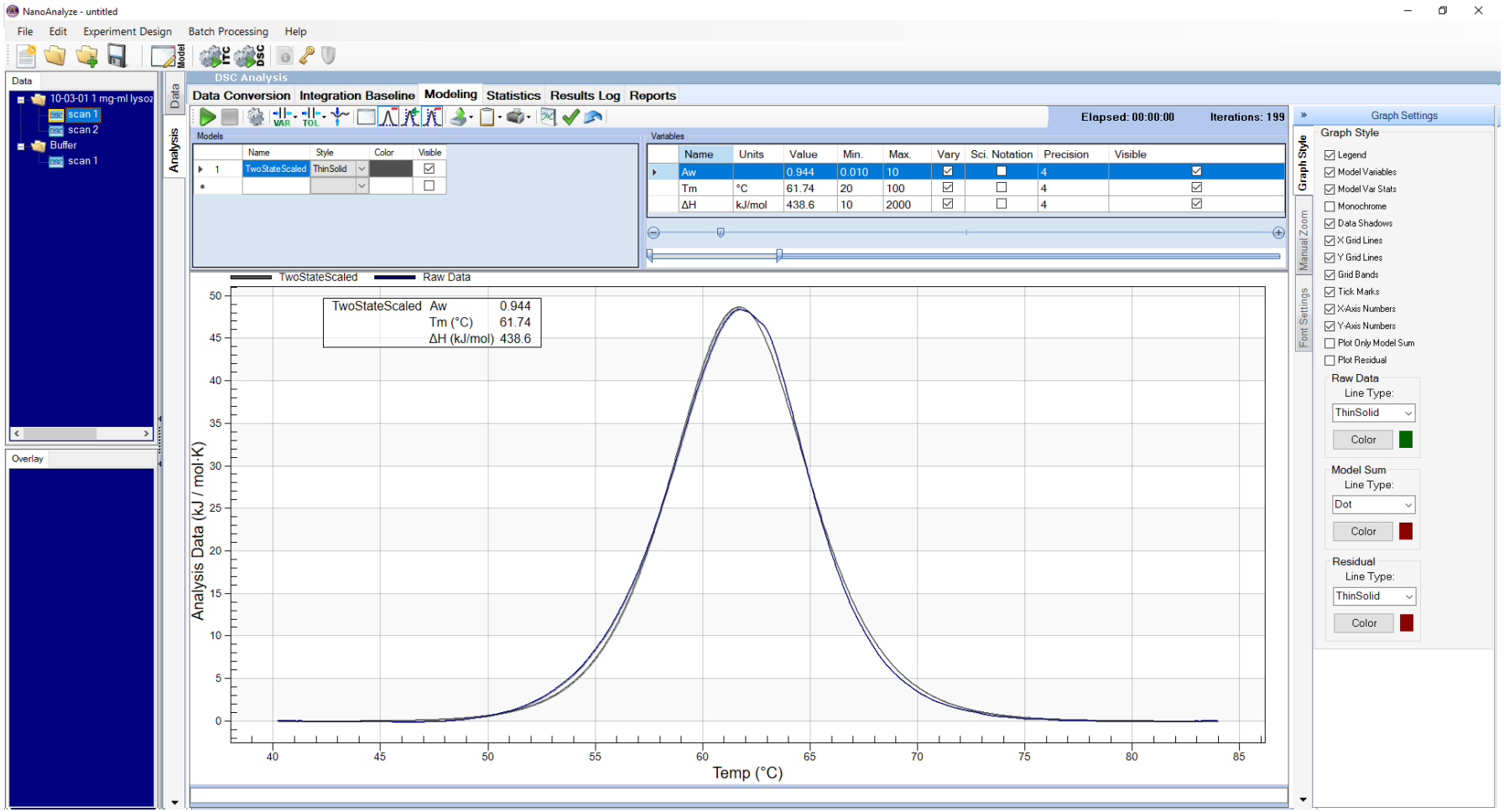
NanoAnalyze – Data Conversion Tab



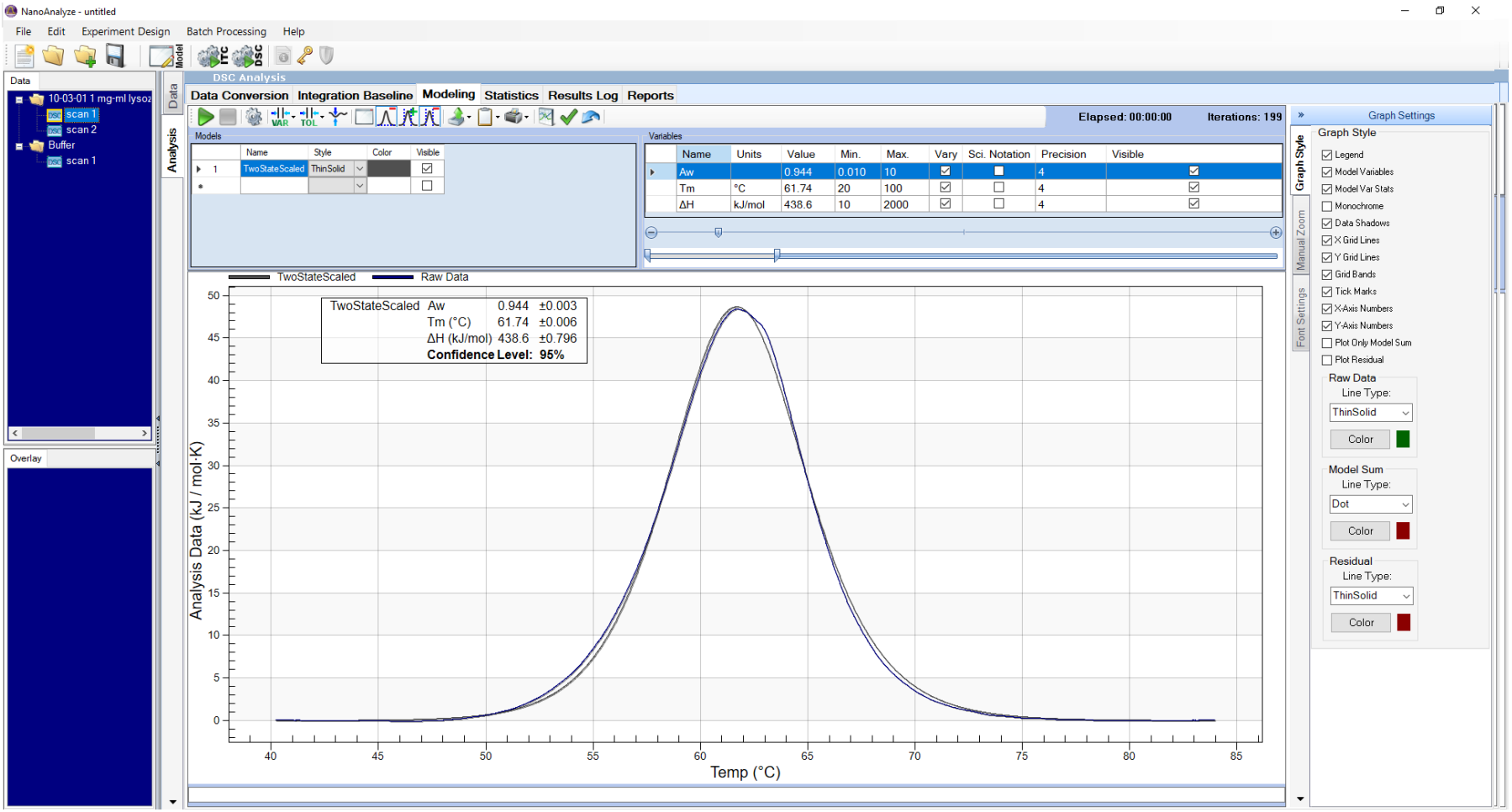
NanoAnalyze – Integration Baseline Tab



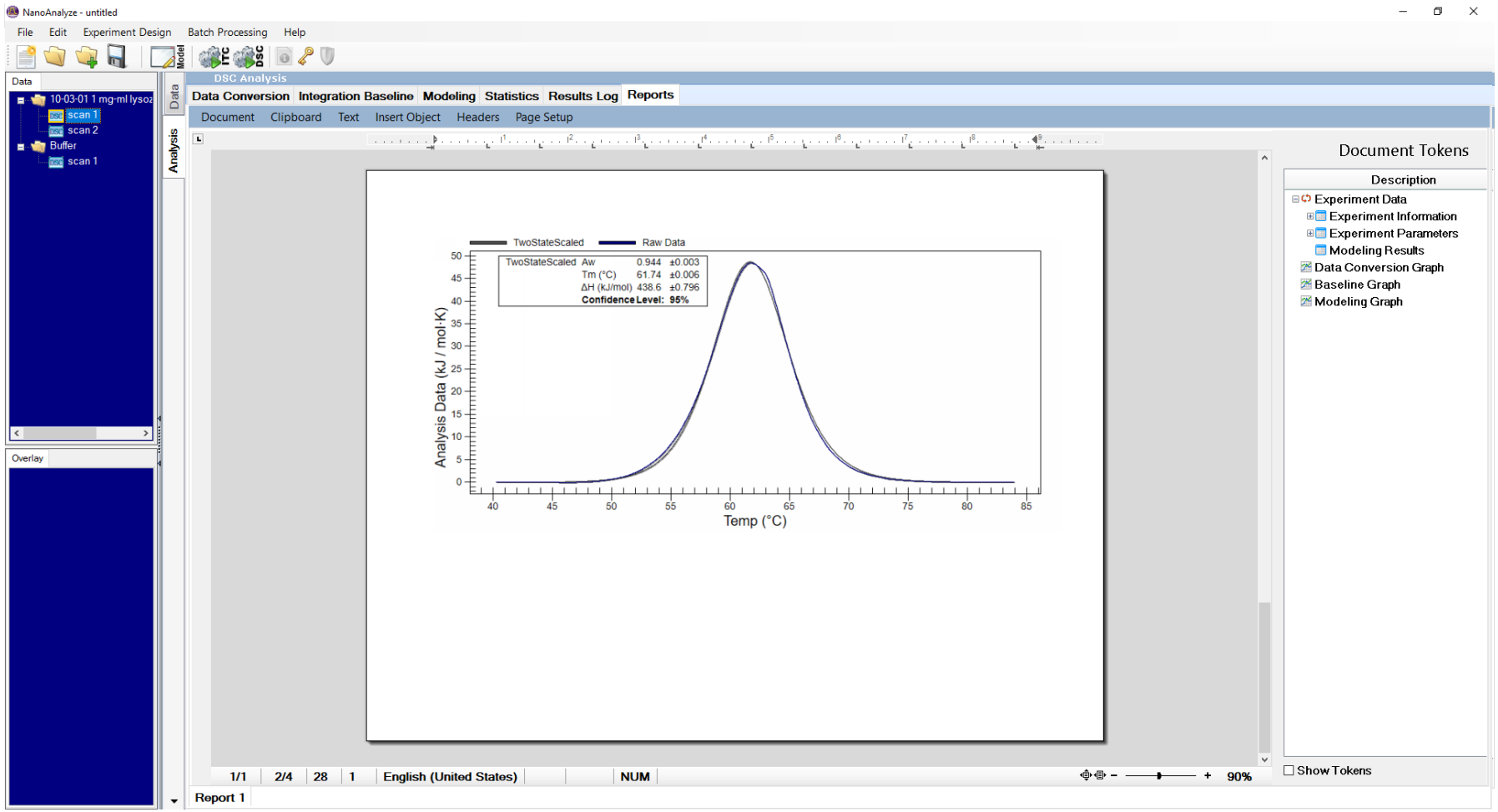
NanoAnalyze – Modeling Tab



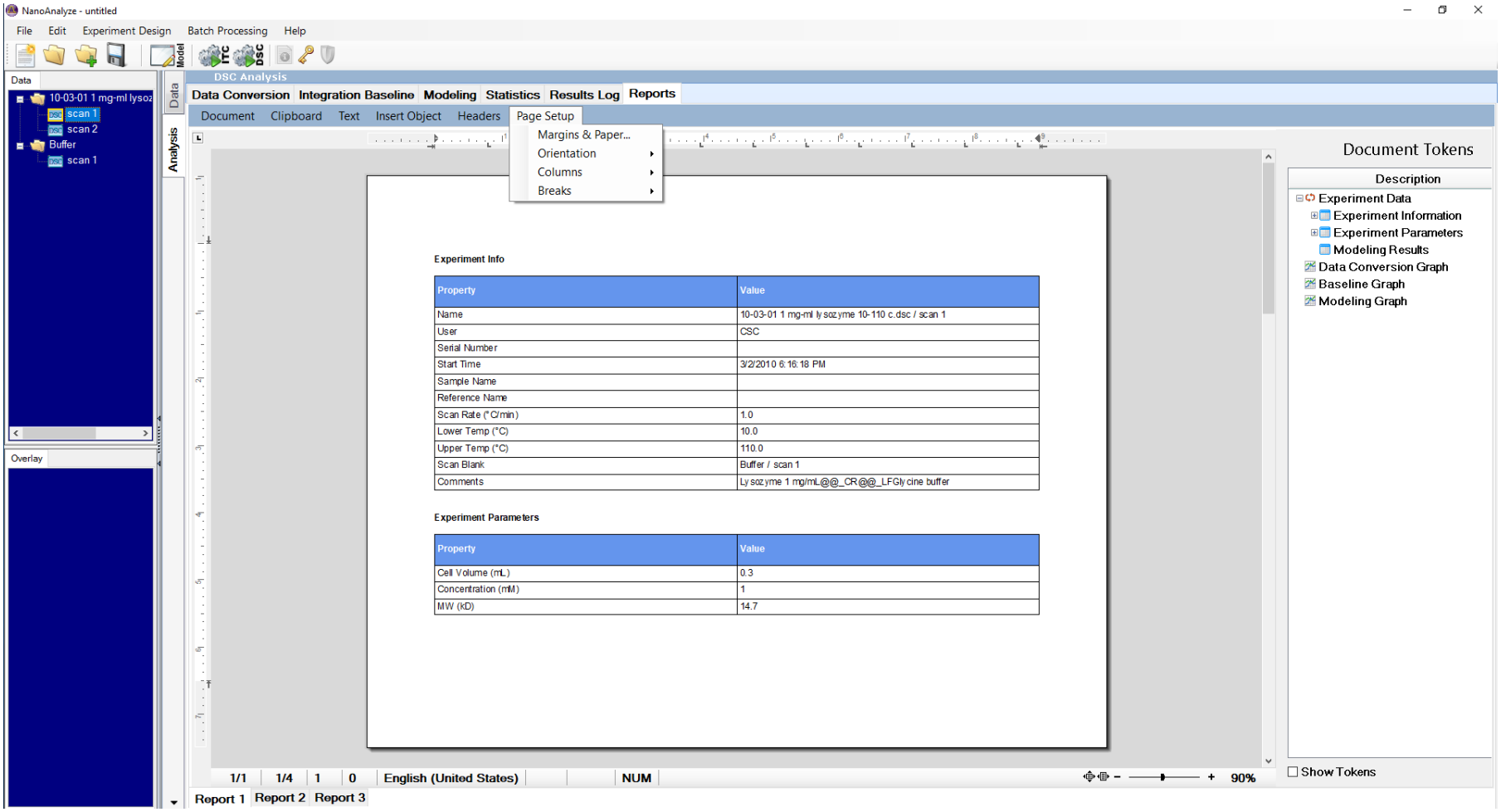
NanoAnalyze – Statistics Tab



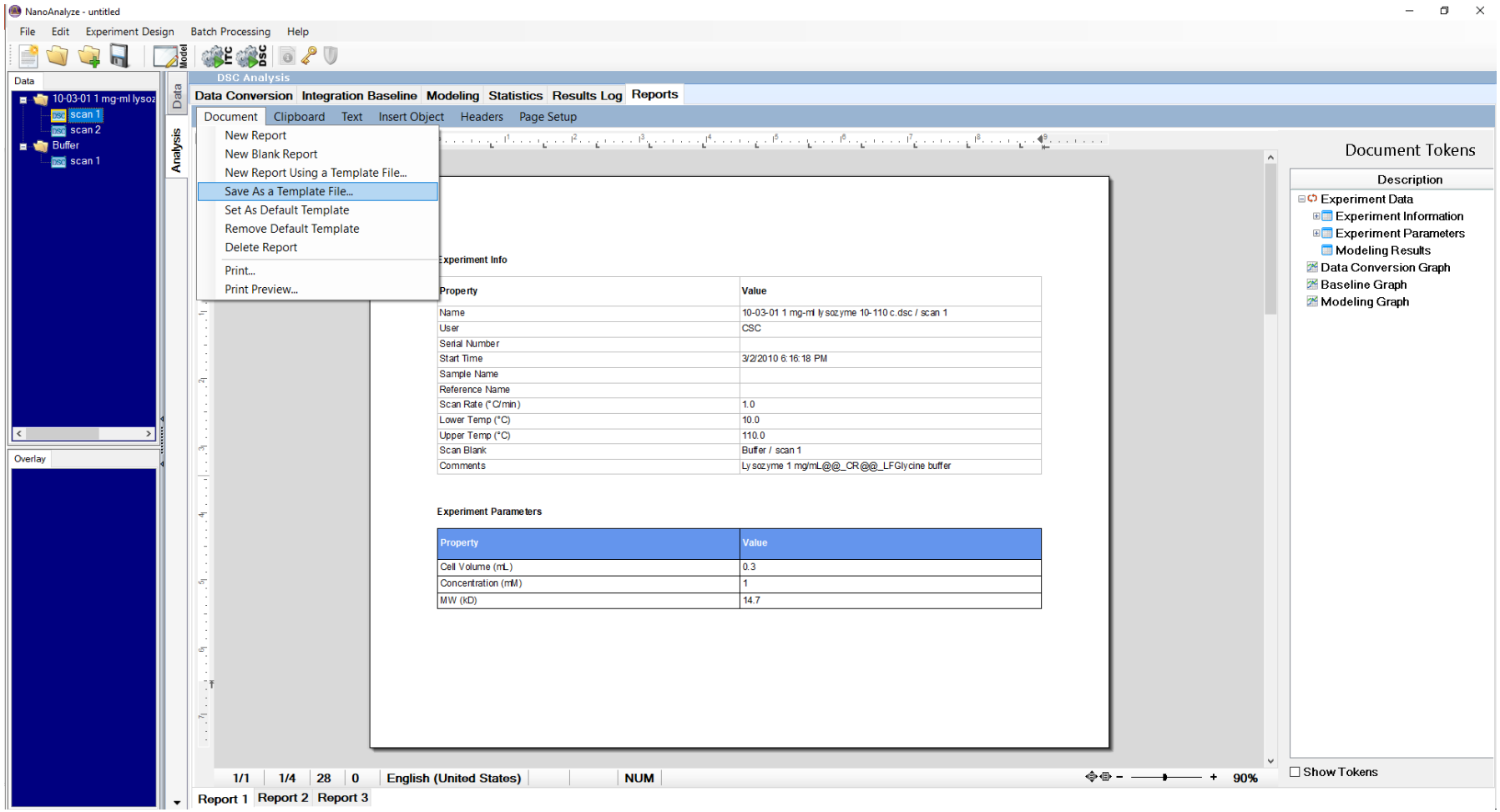
NanoAnalyze – Reports Tab



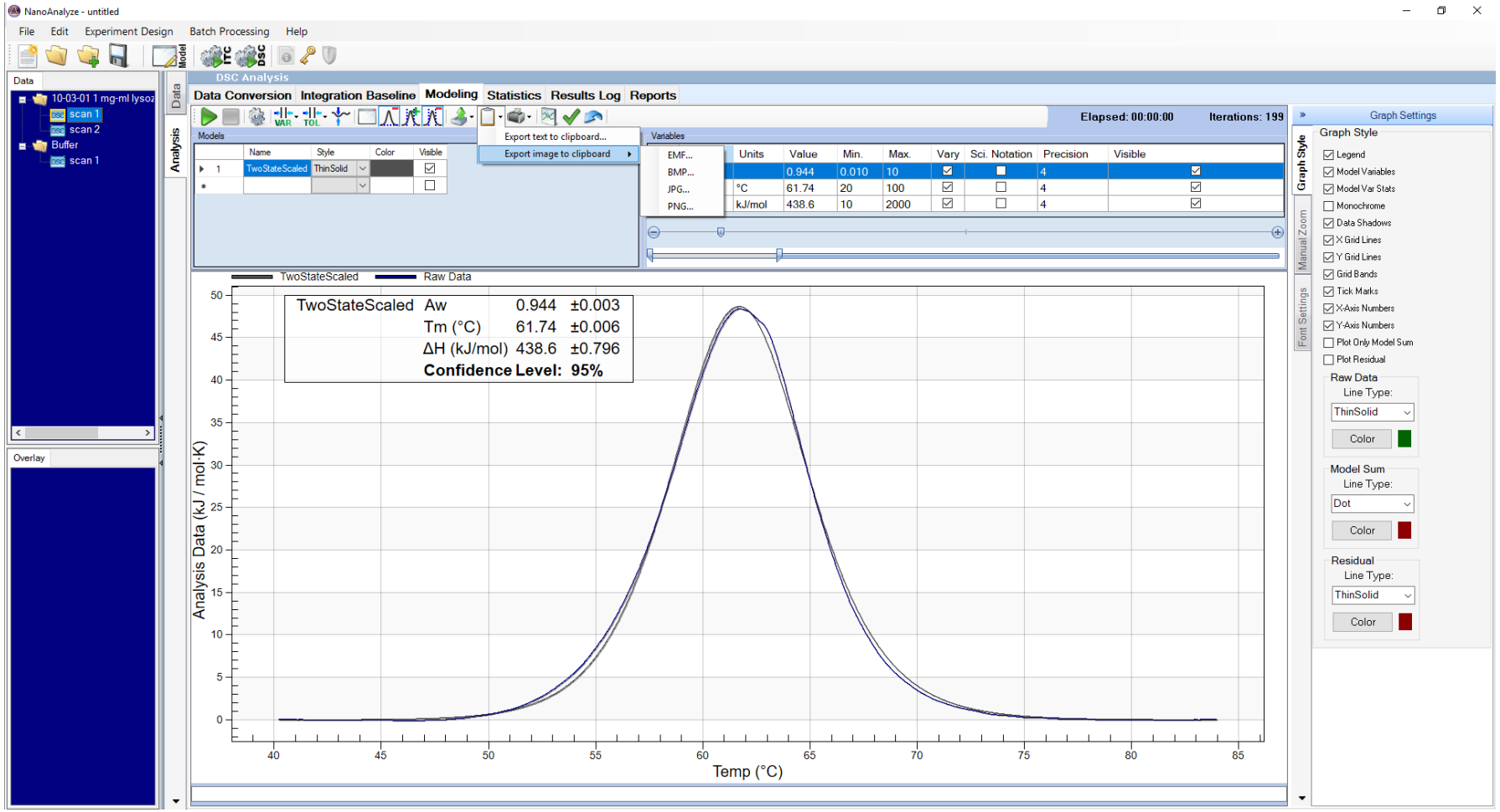
NanoAnalyze – Reports Tab



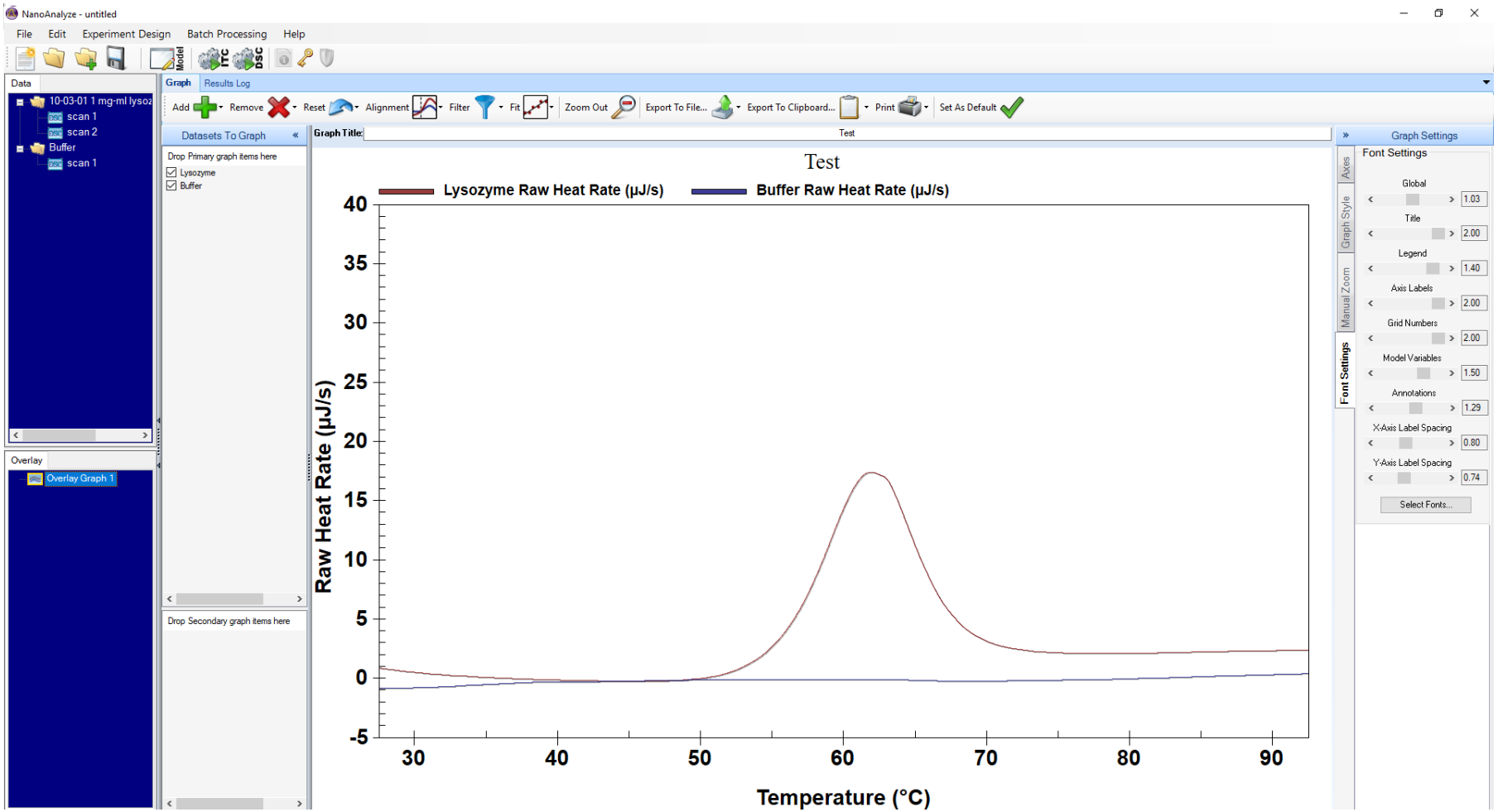
NanoAnalyze – Reports Tab



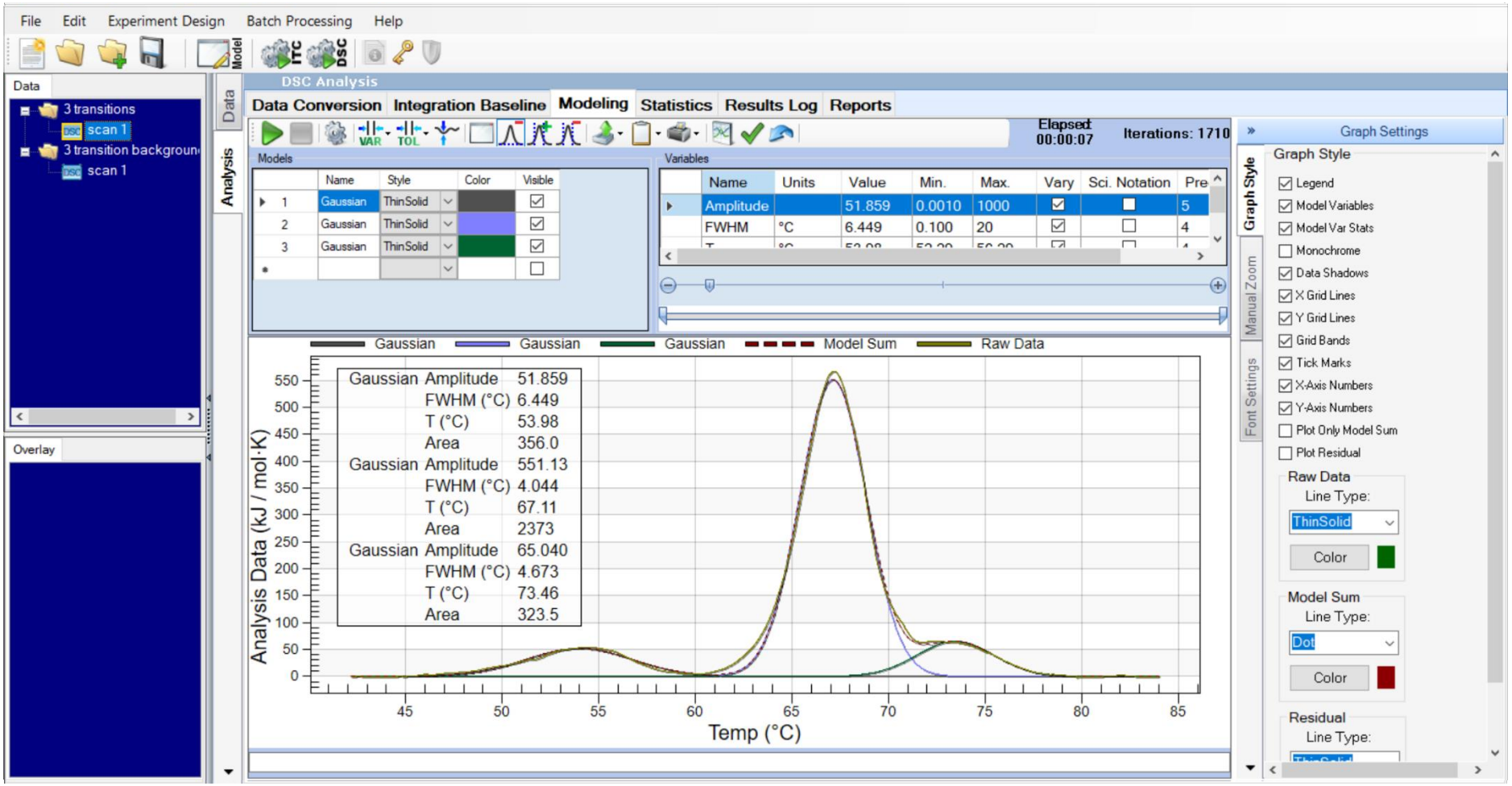
NanoAnalyze – Graph & Data Export Options



NanoAnalyze – Overlay Graph Display



NanoAnalyze – Multi-Transitions



Nano DSC Maintenance

- Laboratory Conditions
  - Environmental Control
  - Line Voltage Conditioning
- Instrument Baselines
  - Balance
- Cell Cleaning
- Pressure Ring
- Fan Filter
- Other Service Support

- From the Nano DSC Getting Started Guide. Available on any computer with DSC Run installed.

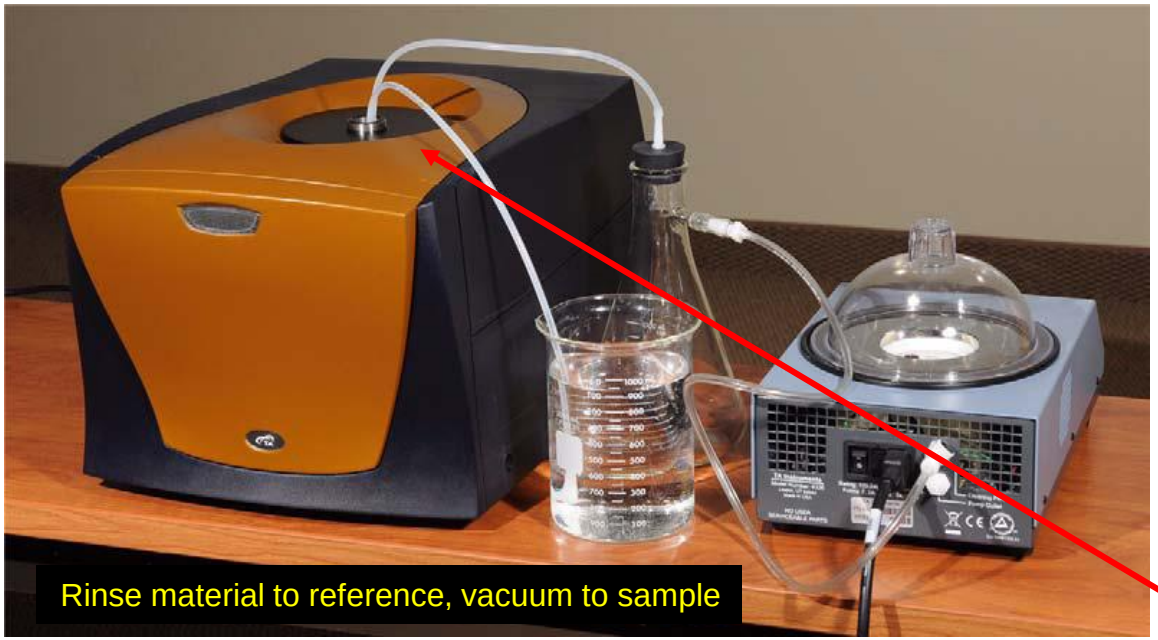
Nano DSC Maintenance

- Instrument Baselines – Software compensation for unique hardware characteristics
  - **Balance**
    - Polynomial correction of the “imbalance” between the sample and reference cells. Instrument delivered with balance scan performed.
    - Not routinely required – contact TA before running
    - Hard-coded parameters: 0 to 130° C at 1° C/minute

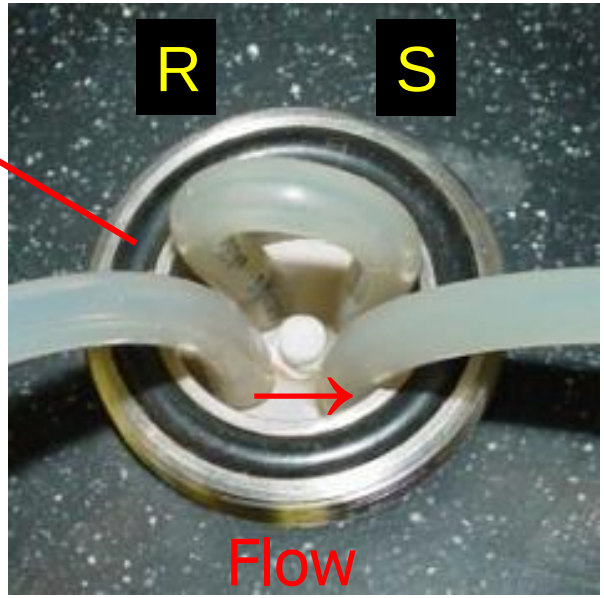
Nano DSC Maintenance

- Cell Cleaning
  - Rinsing with filling pipette or syringe
  - Chemical cleaning
  - High-volume flushing with vacuum degassing accessory

Nano DSC Cleaning Configuration



- Clean cells with 2-5% detergent and/or 15% methanol, rinse
- 4M NaOH at 60-80ºC for 20 min, rinse, treat with 2M formic acid, rinse (1L)



Do NOT contaminate the reference side, cleaning/rinsing flow should move from the reference side to the sample side!

Cell conditioning and buffer baseline scans

- To test cell cleanliness, scan degassed water (heating and cooling) at 3 atmospheres overnight. Baseline should be featureless and reproducible.

- Condition both cells by filling with degassed dialysis buffer, cap one end of each cell, pressurize, and scan (2 ^{o}C/min) to maximum desired temperature.

- Refill both cells with degassed dialysis buffer. Cap one end, pressurize, scan at appropriate rate and temperature range overnight to check baseline repeatability.

Nano DSC Cleaning Procedure

Cell cleaning of the Nano DSC may be performed a number of ways, depending on the extent and nature of the contamination. One typical method is presented here. It may be necessary to perform this cleaning procedure to restore optimum instrument performance.

1. Fill both cells with a 2-5% detergent (Contrad 70) or concentrated solution of sodium hydroxide (~ 4N NaOH). (For more effective cleaning, try a mixture of 4N NaOH containing, 15% methanol and 15% residue-free detergent, such as Contrad 70.)
2. Specify a scan range from 25° C to 80° C. Set the heating scan at 2° C/minute, followed by an isothermal for 20-60min. Longer time may be desired depending on contamination.
3. Abort the scan and allow the Nano DSC to cool to idle temperature (25° C).
4. Remove the cleaning solution from both cells, and flush the cells with ~1 liter of deionized water.
5. Fill both cells with a solution of 50% formic acid, pressurize, and repeat the same scan parameters as before.
6. Abort the scan and allow the Nano DSC to cool to idle temperature (25° C).
7. Remove the acid from the cells, and again flush them with ~1 liter of deionized water.
8. Fill the cells with degassed deionized water and run a scan up and down between 10 and 130° C to condition the cells.
9. Discard the water from step 9 and refill the cells with *degassed deionized water for storage.*

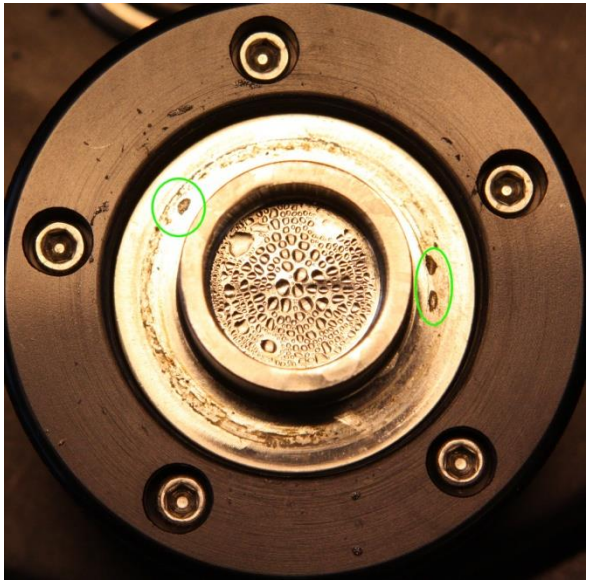
Nano DSC maintenance

- Laboratory Conditions
  - Environmental Control
  - Line Voltage Conditioning
- Instrument Baselines
  - Balance
  - Cell Cleaning
- Pressure Ring
- Fan Filter
- Other Service Support

- From the Nano DSC Getting Started Guide. Available on any computer with DSC Run installed.

Nano DSC Maintenance

- Pressure Ring
  - Cell filling chamber and pressure tube
  - Viton O-ring
  - Pressure Cap – dry off after experiment



- Check the fan filter on the under side of the instrument (Blue colored instruments only)

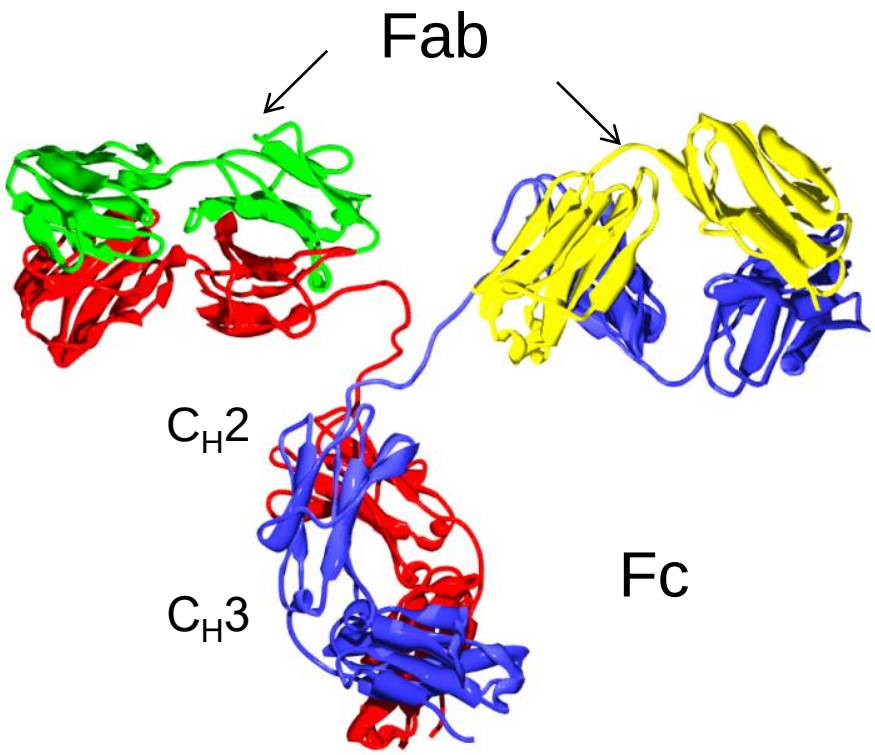
Case Studies

Analysis of Mutated Human IgG1 by Nano DSC and Hydrogen/Deuterium Exchange Mass Spectrometry

Colette Quinn, Ph.D.[1]; Joomi Ahn[2], JiHong Wang[3]

[1]TA Instruments; [2]Waters Corporation; [3]MedImmune

Immunoglobin/Antibody Background

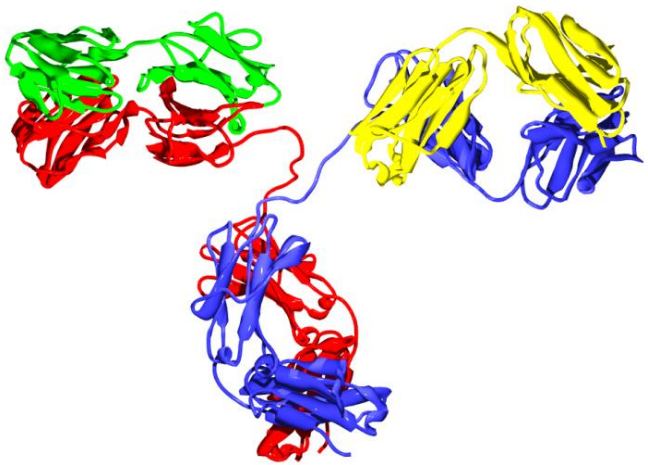


Ribbon Diagram of an IgG antibody. The red and the blue ribbons represent the heavy chains, which define the class of the antibody, and the green and yellow, the light chain portion (2).

- 30 monoclonal antibody (Mab) based drugs approved by US FDA
  - Most IgG1
  - New drug-Mab conjugates
- Japan, Canada, European Medicines Agency already have biosimilar guidelines in place
- Moving beyond Humira and Rituxin to biosimilars
  - Humira – major players Amgen, BI, Fujifilm Kyowa Kirin Biologics, Pfizer
  - Rituxin – 34 biosimilars in development

Objective

- Probe the conformational change caused by a site specific mutation in a hIgG



Ribbon Diagram of an IgG antibody. The red and the blue ribbons (HC) define the class of the antibody, and the green and yellow (LC)

- Analytical Techniques
  - 1.Hydrogen-Deuterium Exchange Mass Spectrometry (HDX MS)
  - 2. Differential Scanning Calorimetry (DSC)

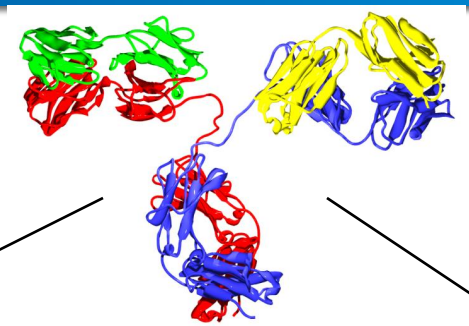
HDX MS Applications

1. Examine Protein Folding
2. Formulation and Stability Testing
3. Identify binding sites (Epitope Mapping)
4. Identify site of Protein Aggregation
5. Mutation Affects

HDX MS Experiments

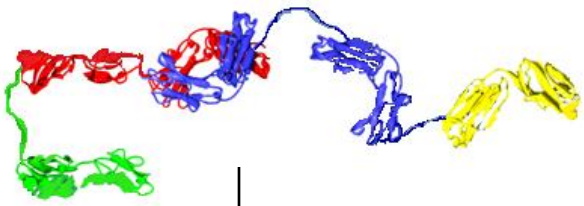
Pulsed Labeling

Continuous Labeling

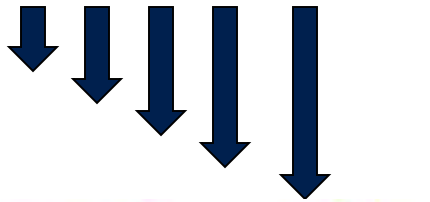


Denaturant +

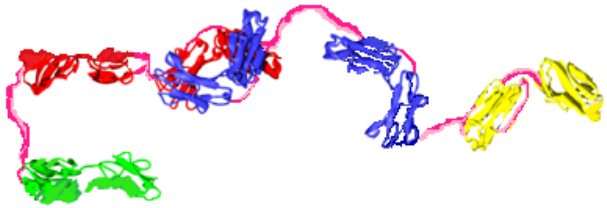
+ D_2O
Physiological pH and 25 ˚C



Multiple aliquot removals



+ D_2O (fixed time)



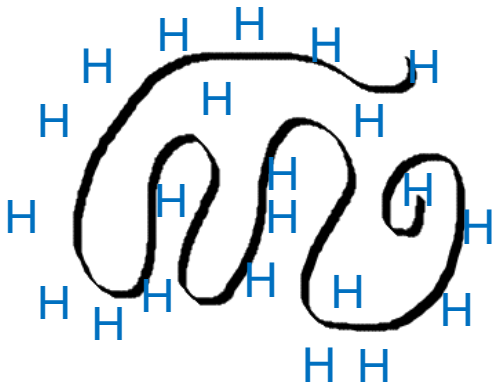
0 ˚C and pH
2.5 Quench

Pepsin digest

Global Overview

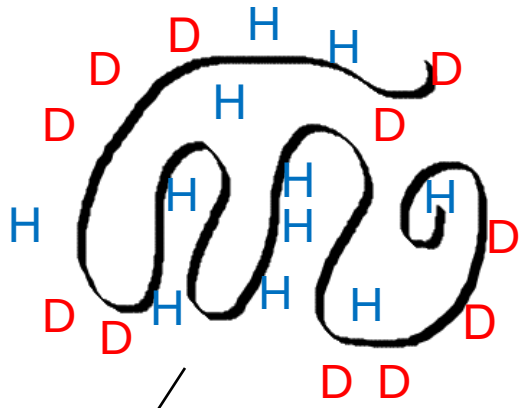
Local Overview

The HDX Experiment



1. H/D Exchange
Dilution in D_2O
Physiological pH, 25 ˚C

Amide H+

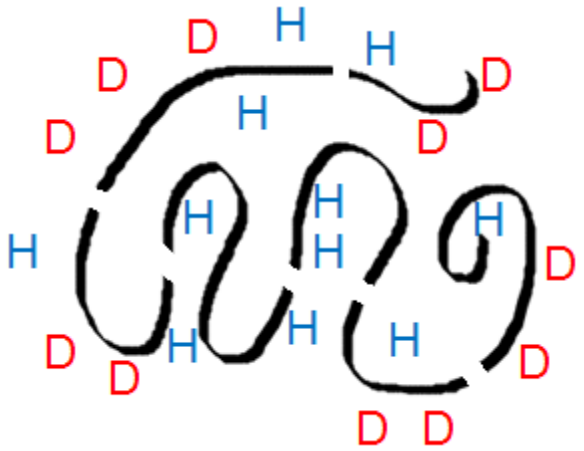


2B. Quench Exchange &
Pepsin Digest
Low pH, 0 ˚C

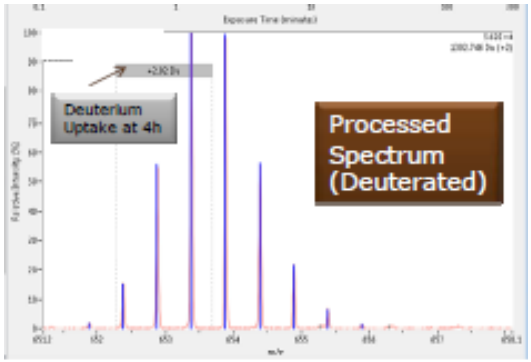
Local

2A. Quench Exchange
Low pH, 0 ˚C

Global



3. Liquid Chromatography &
Mass Spec
Low pH, 0 ˚C

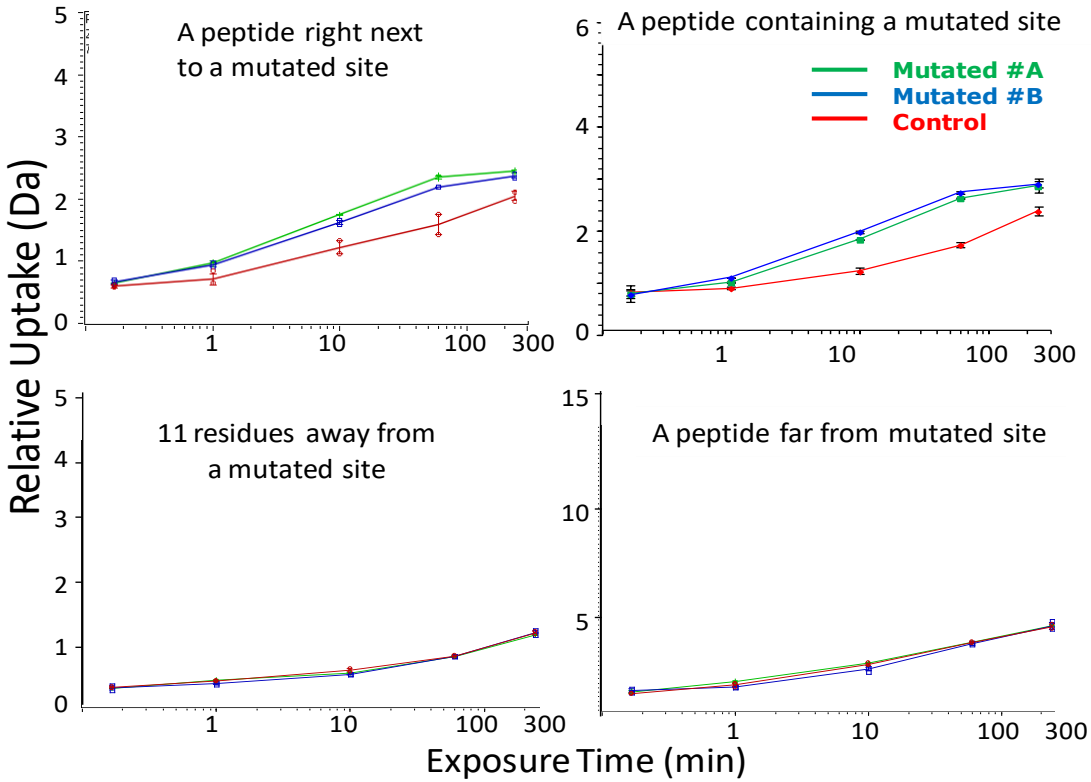


HDX Uptake Curves
Local Path

HDX

Changes

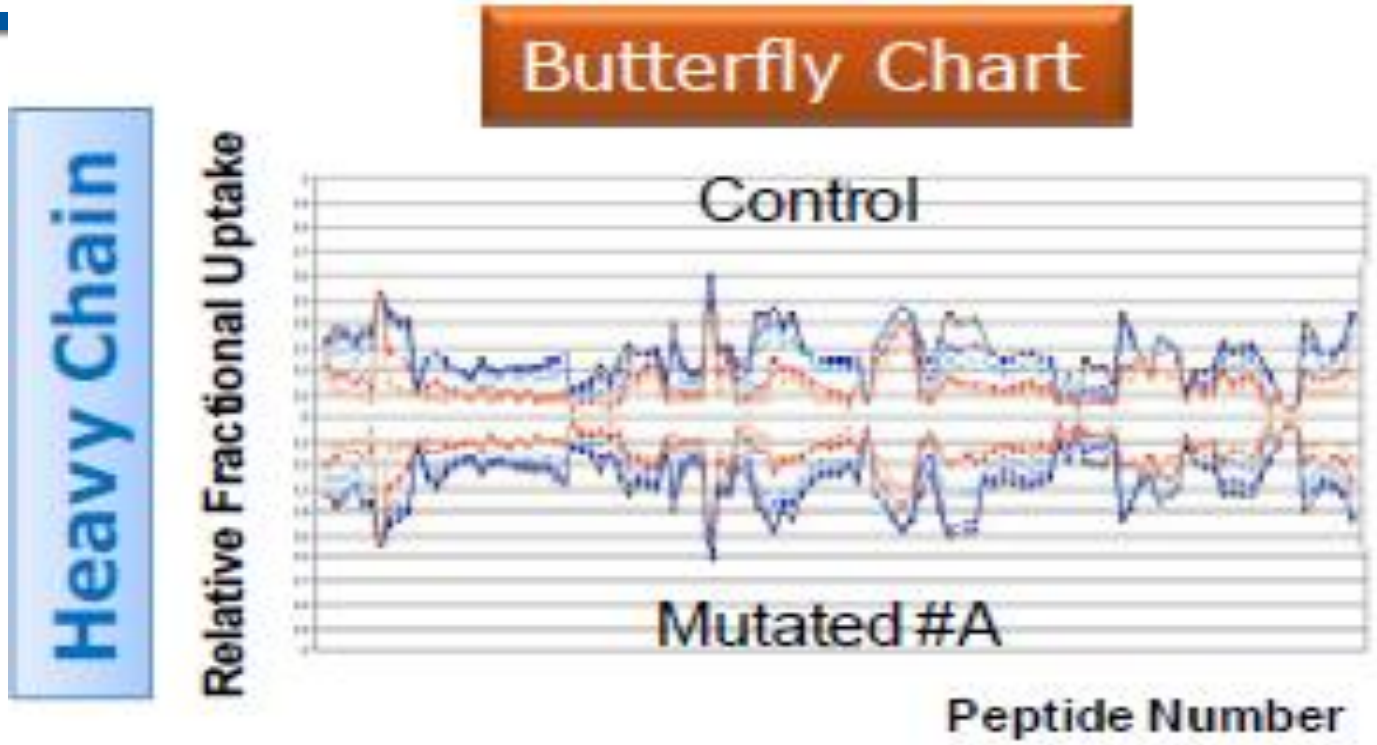
No Changes



•Increased D uptake of the peptides indicates that the mutated region is more flexible compared to the control antibody.

The deuterium uptake curves from the HDX MS study with (top two panels) and without changes (bottom two panels) comparing the control to the mutated samples for four selected peptides.

HDX Results

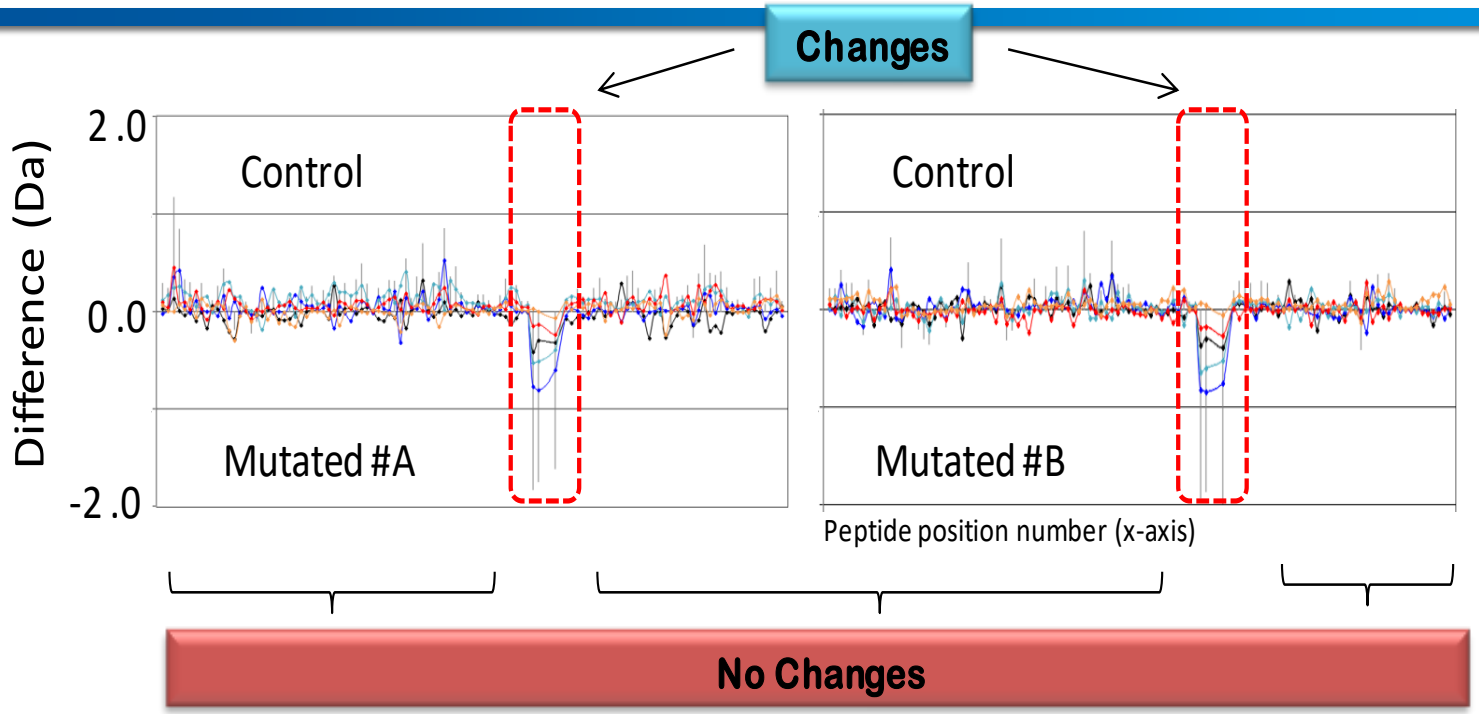


•Each point indicates the difference in uptake for a specific peptide between the control and a mutated sample.

•Each exposure time is in a different color – providing a line that represents the uptake for all of the peptides at that time

•1 IgG, 230 peptides, 6 time points, done in duplicate = over 2700 spectra

DynamX™



DynamX "Difference" plots comparing the mutated IgG #A and mutated IgG #B to the control in the left and right panels, respectively. The red boxes highlight the peptides near and at a mutated site.

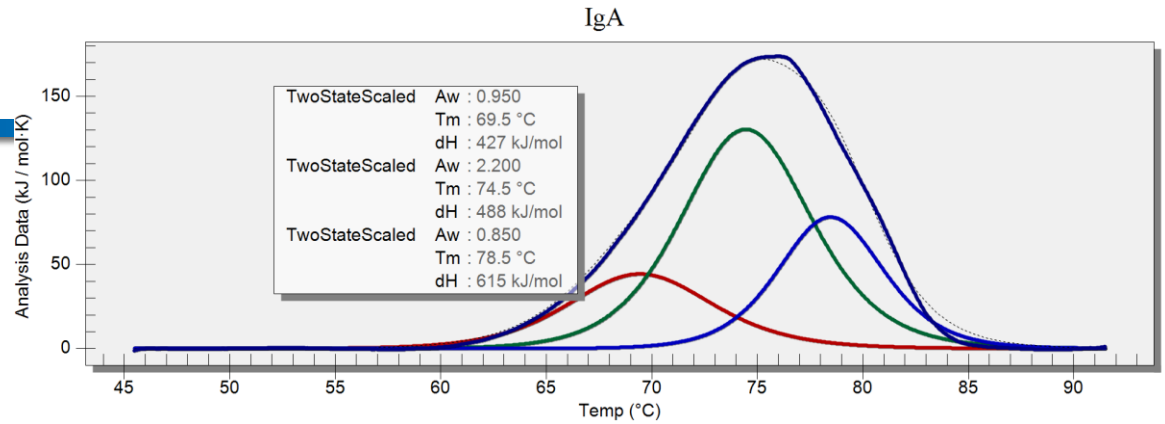
- The vertical bar represents the sum of the uptake differences across the time-points
  - Longer vertical bar, the larger difference
  - Vertical bars in indicate statistically significant difference

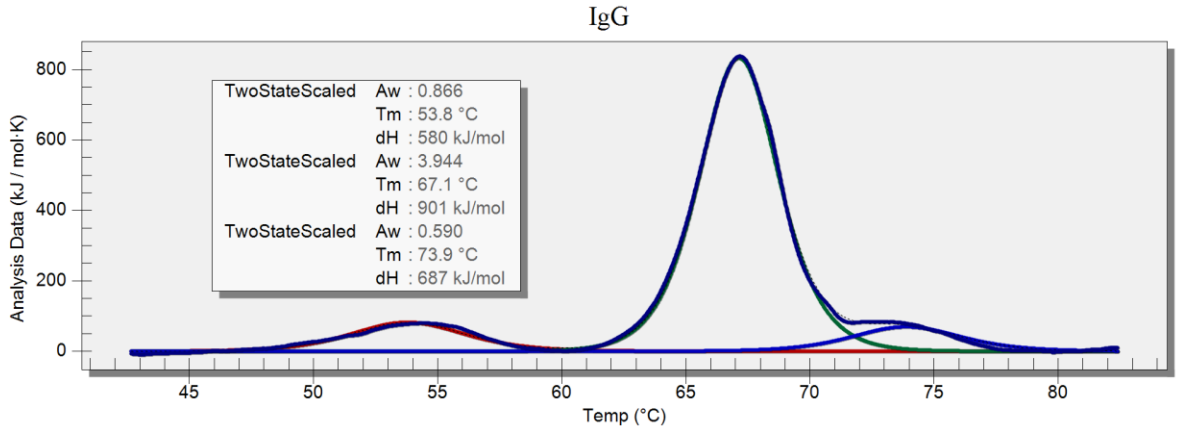
DSC Experimental

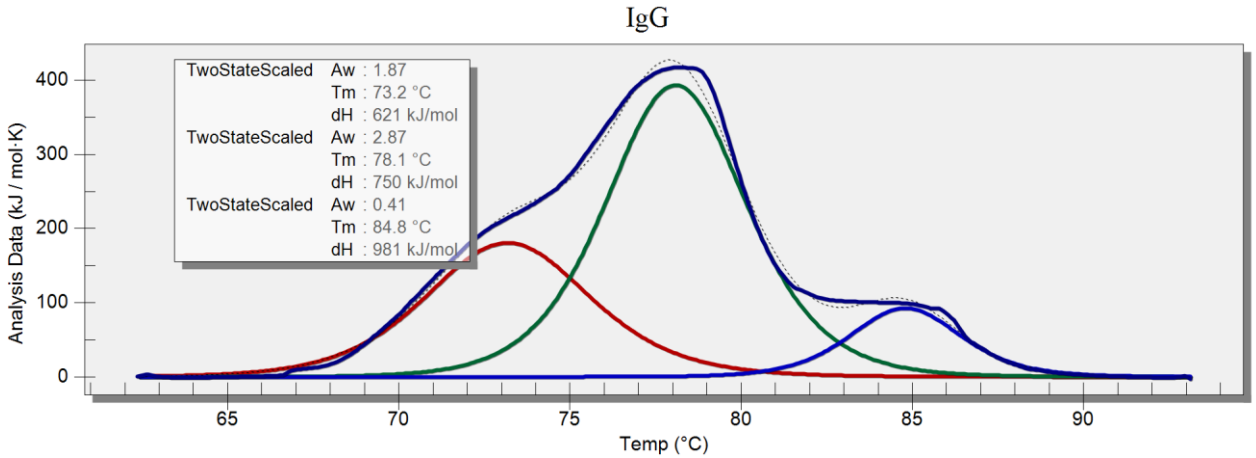
1. 900* µL of protein in sample well, 900µL of buffer into reference well
2. Auto sampler at 4 ˚C
3. Scan sample from 25 to 100 ˚C at 1 ˚C/min
4. All data was background corrected with a buffer into buffer scan under identical conditions.

*current volumes used are 600 µL

Hinge Region

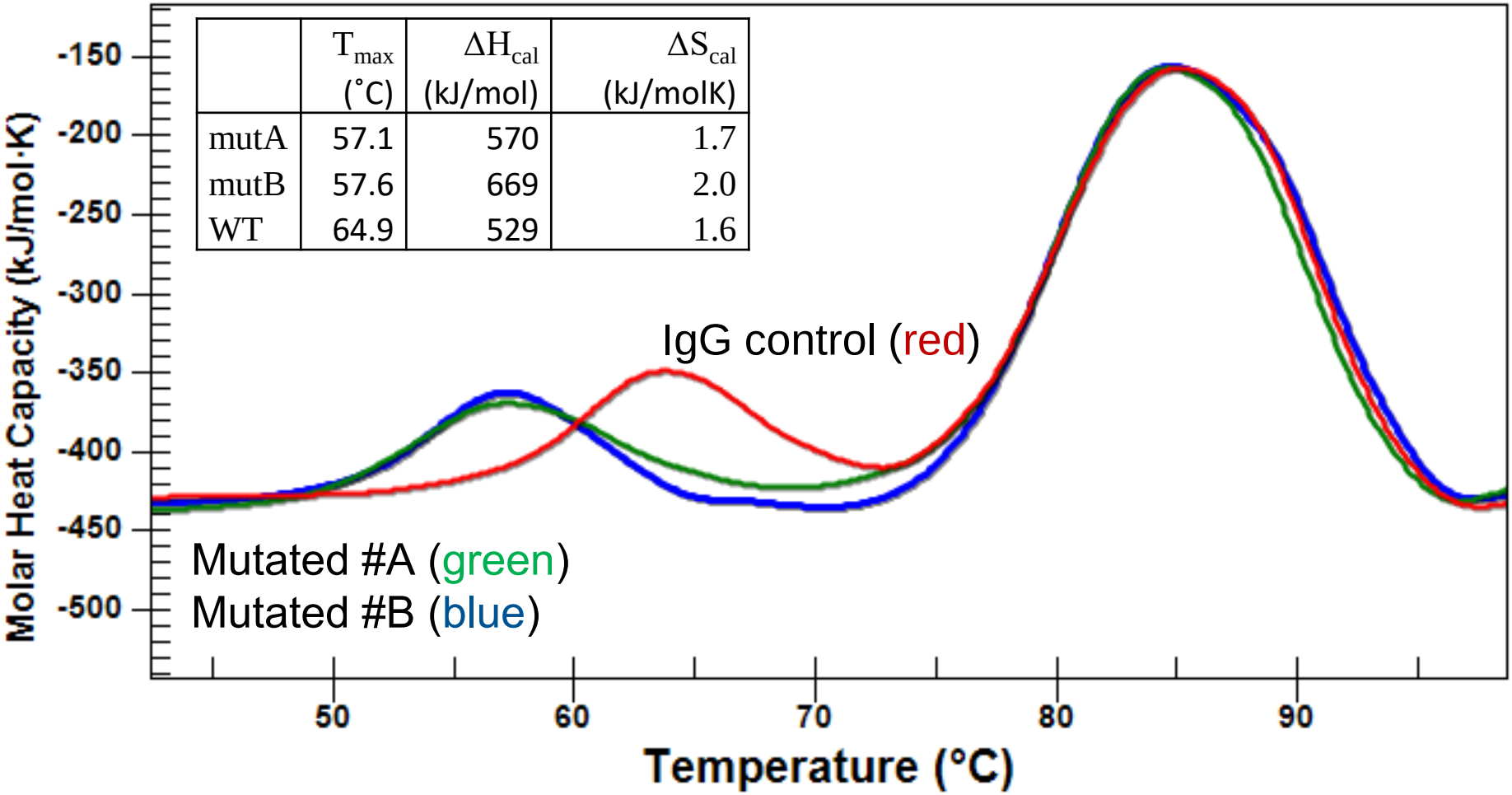






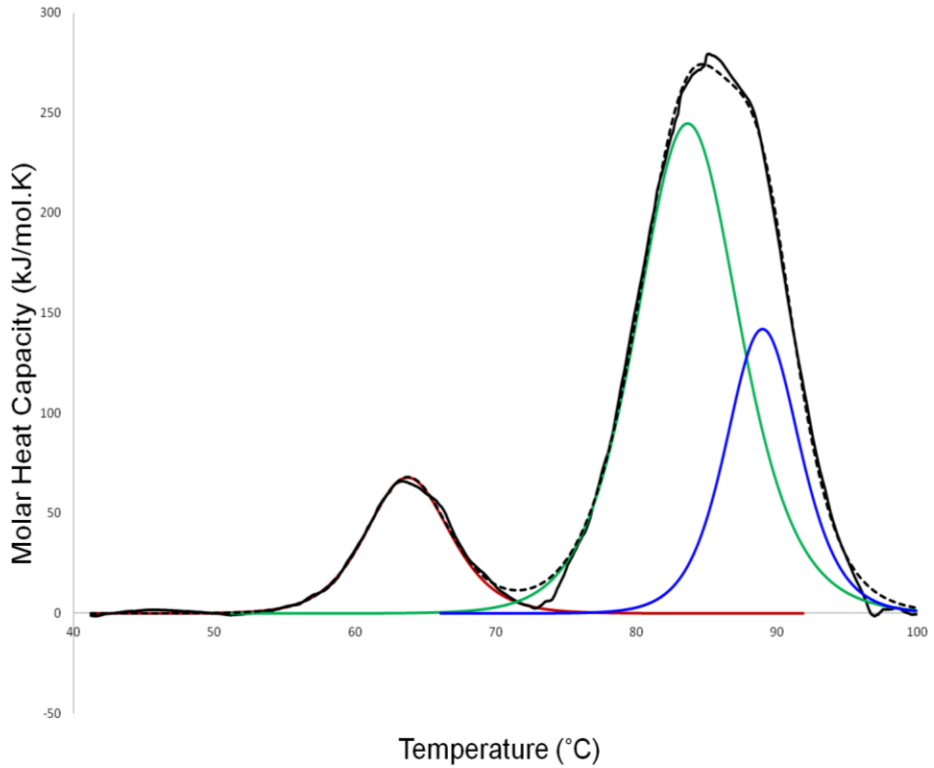
Nano DSC Results

	T_{max} (˚C)	ΔH_{cal} (kJ/mol)	ΔS_{cal} (kJ/molK)
mutA	57.1	570	1.7
mutB	57.6	669	2.0
WT	64.9	529	1.6



Identification of domains: Wen, J.; Jiang, Y. *American Pharm. Rev.*, **2008**, 11, 98. - fragmentation study

Modeling Data and Thermogram Deconvolution



- The fitting algorithms were able to successfully and accurately deconvolute this broad, asymmetrical, unfolding event.

- DSC thermogram (black) fitted to three events (red, green and blue) the sum of the three fits is indicated by a dashed black line

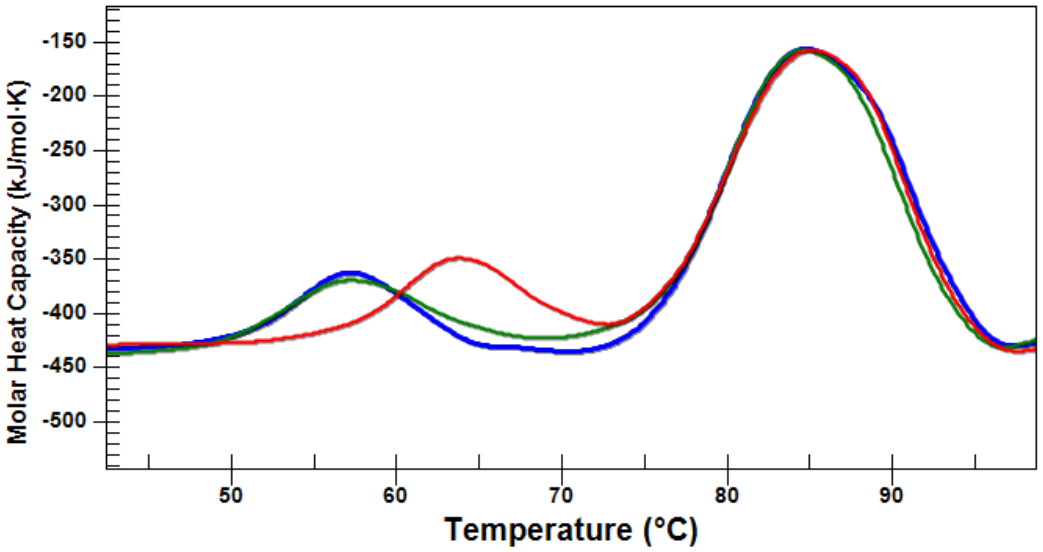
General Analysis of Mutation A, B and Control

	Q_1 (kJ/mol)	T_{m1} (˚C)	Q_2 (kJ/mol)	T_{m2} (˚C)	Q_{total} (kJ/mol)
Mut. #A	526 (15%)	57.5	2983 (85%)	85.1	3509
Mut. #B	654 (16%)	57.8	3520 (84%)	85.3	4174
Control	506 (13%)	65	3227 (87%)	85.5	3792

- First peak, definite change. Second peak there was not a significant change.
- The mutation and its effects were isolated.
- Q% are consistent with the mass ratio of domains. With this simple comparison it is more easily observed that the heat related to the unfolding of the first domain has changed.

DSC interpretation

- Number of peaks in antibody is related to the flexibility of the hinge region
- Change in the C_H2 domain does not have a destabilizing effect on Fab or C_H3
- $\Delta H_{unfolding}$ is related to formation, breaking or distorting bonds



Summary

- Differences detected by HDX in deuterium uptake and by DSC in the structural stability revealed the *discriminatory power* of each technique.

- Analysis – Beyond fitting data, understanding.
  - HDX - structural change, flexibility of amide backbone
  - DSC - destabilization of a specific region of the molecules.

- Multi-parameter analysis, critical structure-function relationship for these IgG molecules.
  - Early development process of new biotherapeutics quality control processes that must be in place to produce them.

  - HDX and DSC are two very sensitive and reproducible techniques that should be considered for the detection and accurate characterization of both structure and function.

DSC Case Study:
Antibody drug conjugates

Antibody Drug Conjugates

Pharma interest surges in antibody drug conjugates
Webb, S. Nature Biotechnology 29, 297–298 (2011)

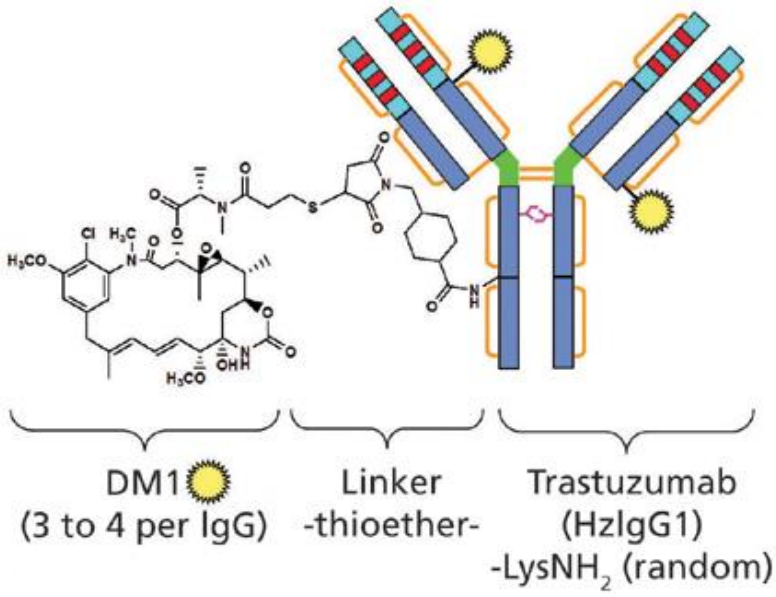


Figure 1: Structure of trastuzumab emtansine (T-DM1). Discovery Medicine, A. Beck et al., Discov. Med. 10 (53), 329–339 (2010).

Trastuzumab (Herceptin®) is approved for use only in human epidermal growth factor receptor HER2-positive cancers, but not all HER2-positive cells have sufficient apoptotic capacity to be killed by trastuzumab binding alone

Herceptin targets HER2 receptors in breast and stomach cancer, with DM1 (maytansine) - a small-molecule cytotoxin that binds to tubulin to prevent microtubule formation.

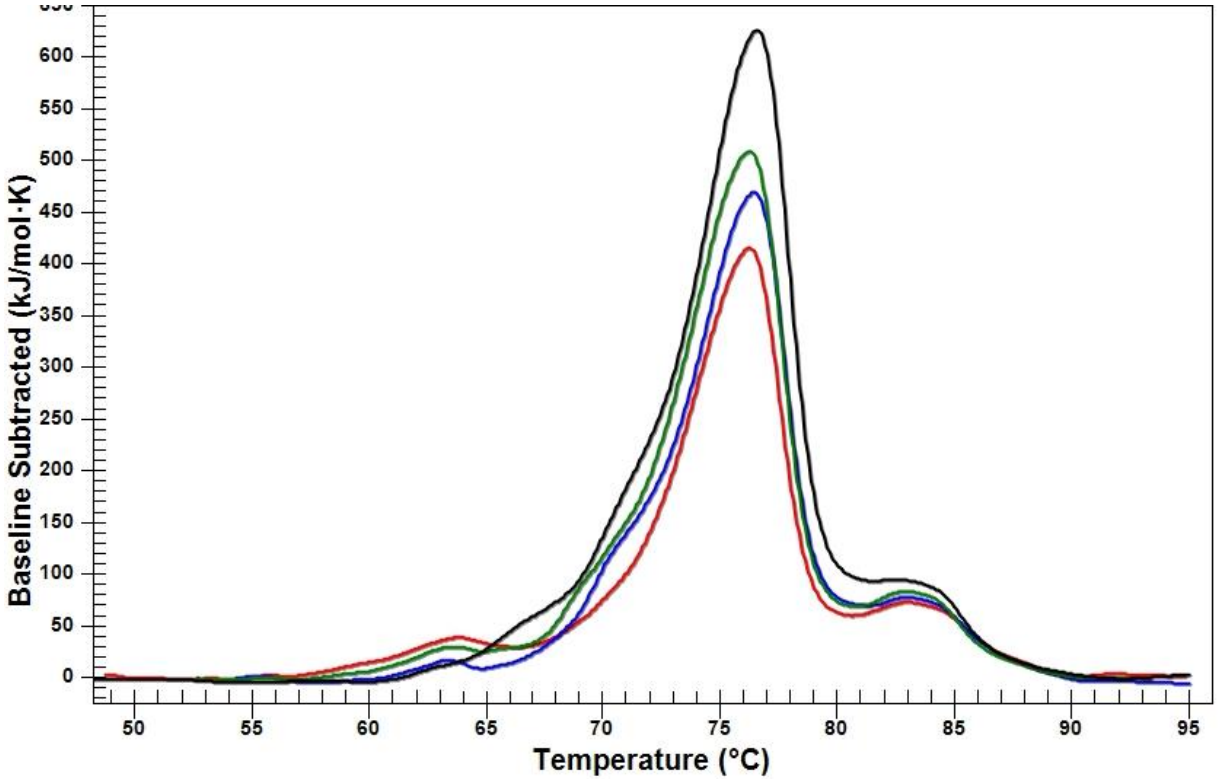
Conjugation Type-1 ADC Series, 0.5 mg/mL

Type-1 High Baseline Substracted (KJ/mol·K)

Type-1 Low Baseline Substracted (KJ/mol·K)

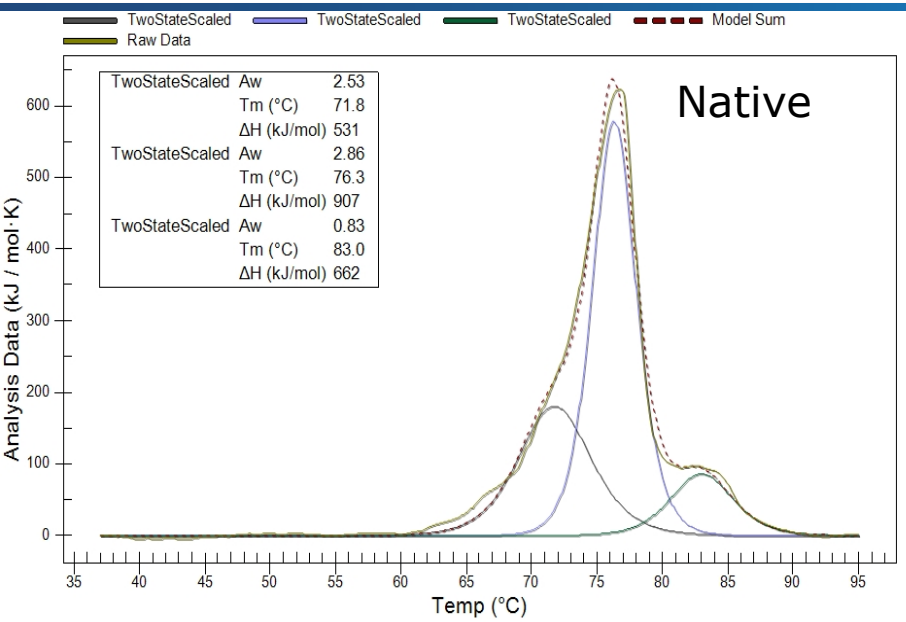
Type-1 Mode Baseline Substracted (KJ/mol·K)

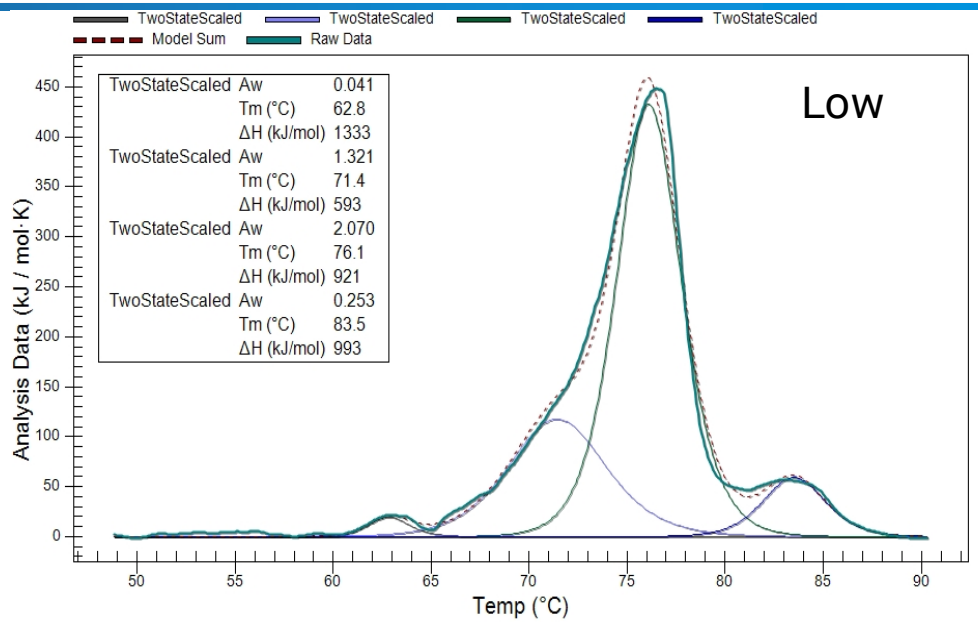
Type-1 Native Baseline Substracted (KJ/mol·K)

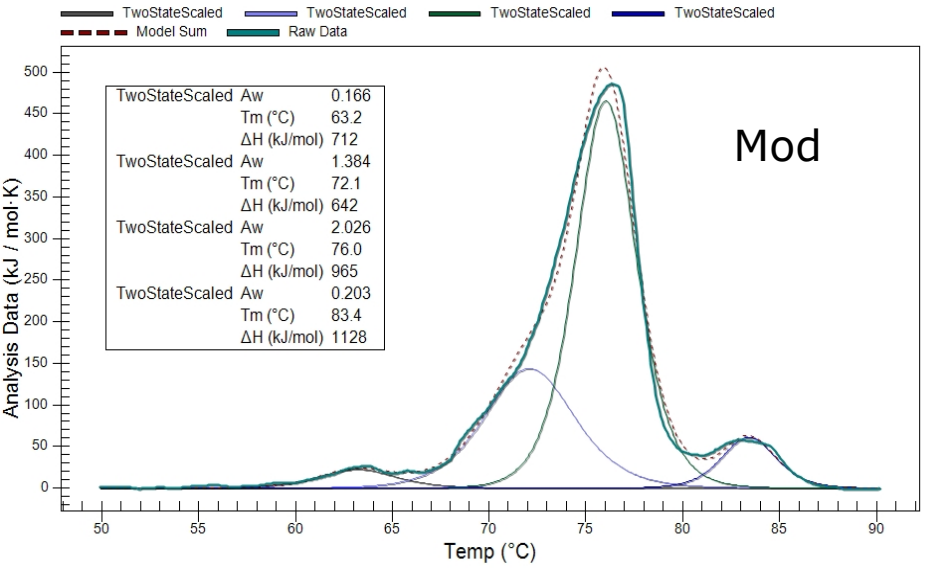


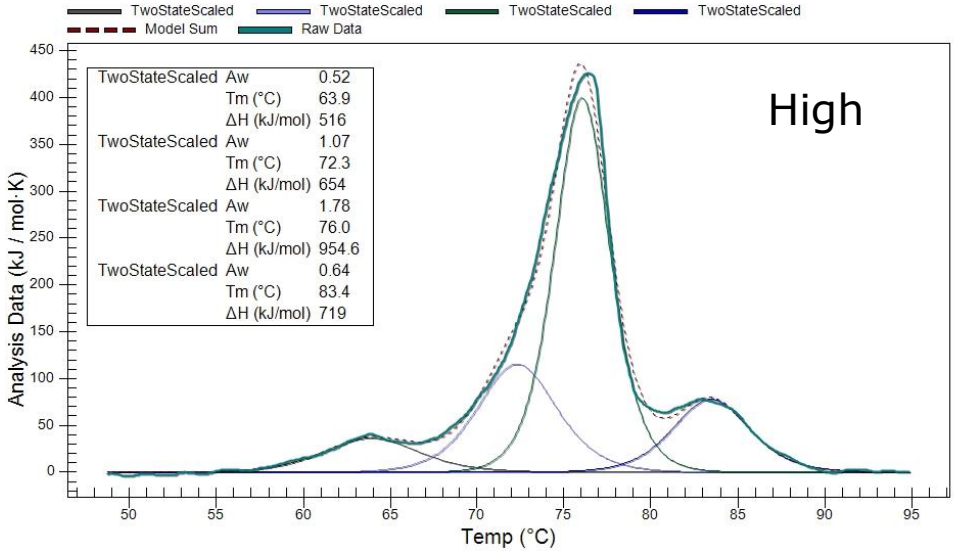
Type-1 SERIES AVERAGES	FWHM	T_{max}	T_{onset}	T_{max}-T_{onset}	ΔH_{cal}	% Enthalpy Compared to Native
Type-1 Nat	5.1	76.7	69.0	7.7	4281	
Type-1 Low	4.8	76.5	69.2	7.3	3321	78
Type-1 Mod	4.9	76.4	69.9	6.5	3461	81
Type-1 High	4.5	76.2	70.5	5.7	2941	69

Fitted Conjugation Type-1 Data

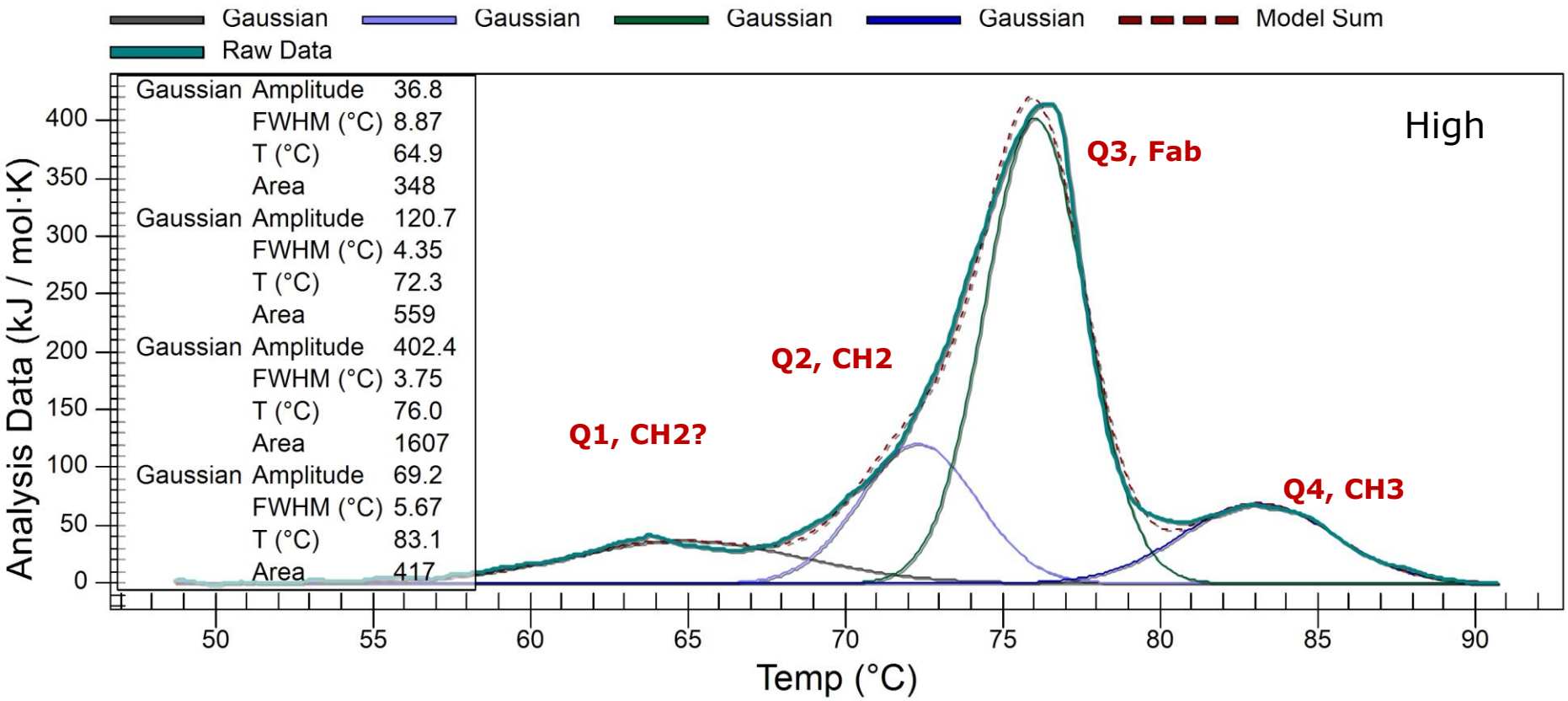








Fitted Conjugation Type-1 Data, relation to antibody regions



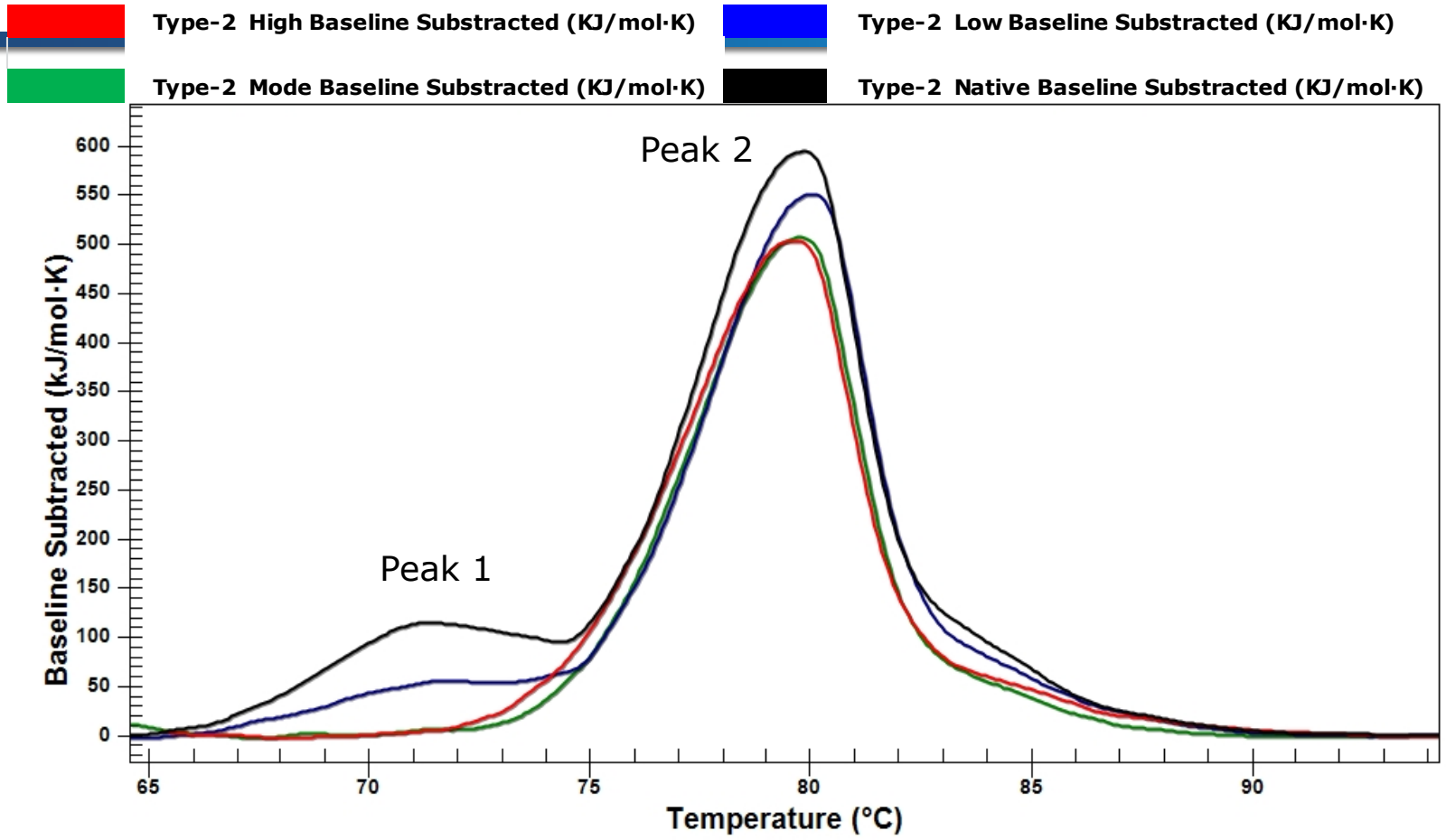
Comments on Fitted Conjugation Type-1 Data

- Deconvolution of the data and subsequent fitting with the pseudo van't Hoff model allows assignment of percentage of heat, Q, coming from each feature($Q = \Delta H_{VH} * A_w$).
- Normalizing the heat allows a comparison across the series.

	%Q1	%Q2+3	%Q4
Native	0	88	12
Low	2	90	8
Mod	4	89	7
High	9	77	15

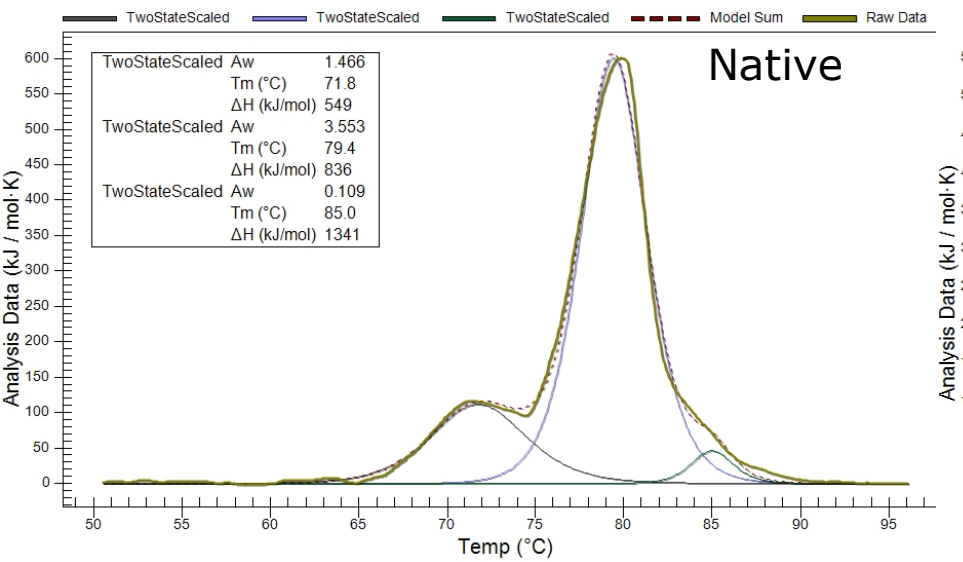
- The majority of the heat originates from the middle temperature transition.
- The low temperature event (Q1) increases from non-existent at Native to 9% at high.
  - The magnitude of heat values tend to trend with the quantity therefore it is reasonable to assume that the population of the this less stable species increased by 9%.
  - The increase in Q1 is linear.

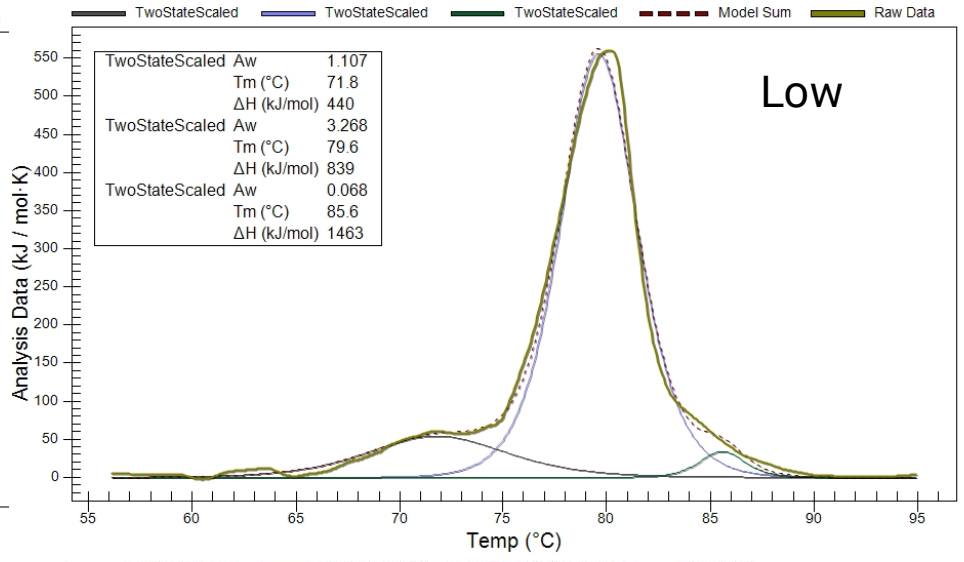
Conjugation Type-2 Series

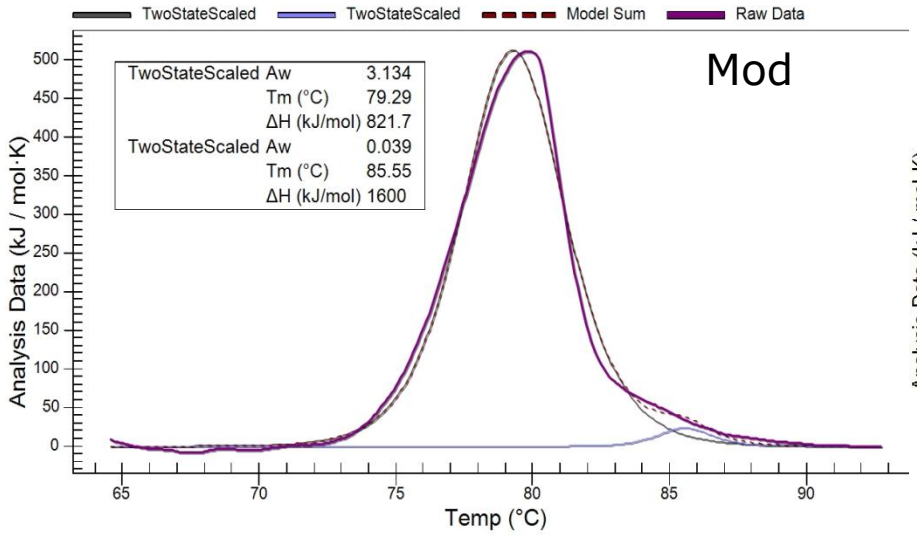


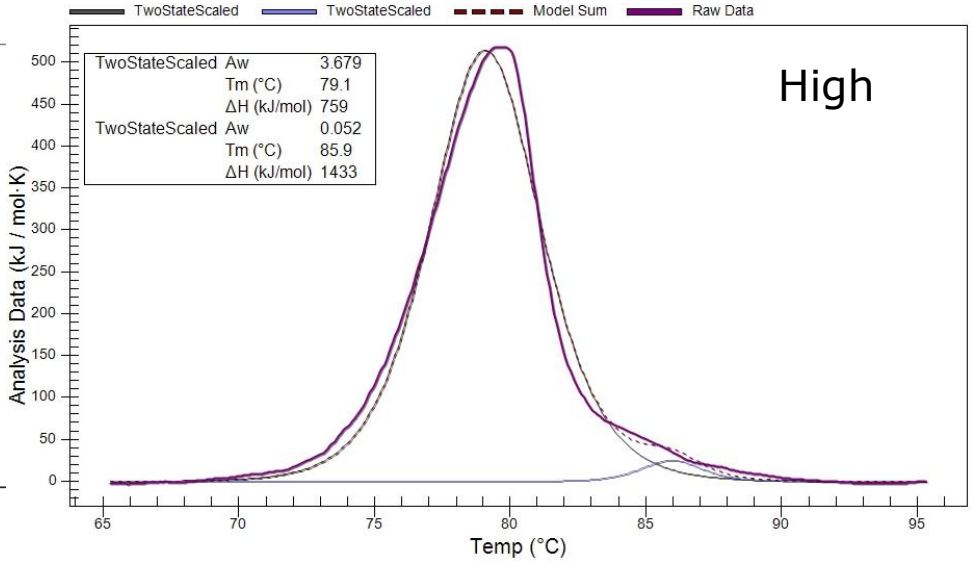
Type-2 SERIES AVG	FWHM Peak 1	T_{max1}	%Area peak 1	FWHM Peak 2	T_{onset2}	T_{max2}	$\Delta H_{cal\ (kJ/mol)}$	T_{max2}-T_{onset2}
Type-2 Nat	6.7	71.8	21	4.8	75.4	79.9	3989	4.5
Type-2 Low	7.0	72.0	15	4.8	75.5	80.0	3370	4.6
Type-2 Mod(1)	-	-	-	4.7	74.9	79.9	2811	5.0
Type-2 High(1)	-	-	-	4.9	74.3	79.6	2728	5.3

Fitted Conjugation Type-2 Data









Comments on Fitted Conjugation Type-2 Data

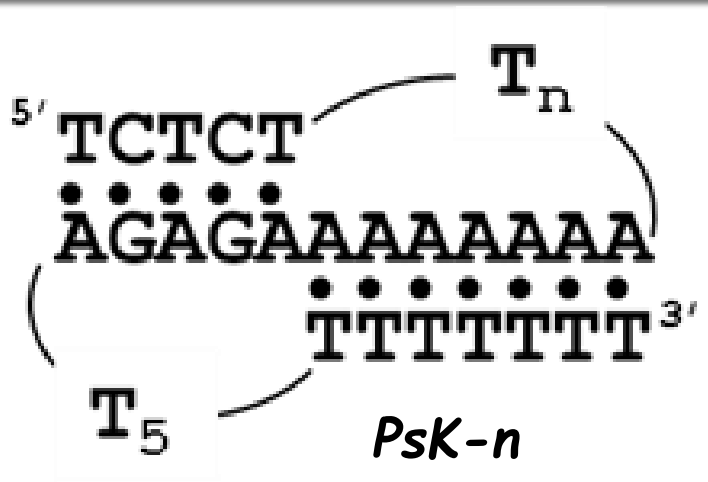
	%Q1	%Q2	%Q3
Native	21	76	4
Low	15	82	3
Mod	0	98	2
High	0	97	3

- The majority of the heat originates from Q2 with an increase the %Q2 as the extent of modification increased.
- This increase was concurrent with an increase in the width which could indicate that the extent of Conjugation Type-2 modification actually stabilized this population.
- For certain antibodies the initial peak can indicate flexibility of the hinge region. It is possible that this flexibility decreases as the modification increases.

DSC Case Study:
DNA Pseudoknot Base-Triplet Formation

Calliste Reiling and Luis A Marky "The Complementarity of the Loop to the Stem in DNA Pseudoknots Gives Rise to Local TAT Base-Triplets." *Methods Enzymol*. 2015

Sequences of Intramolecular Pseudoknots and Control Molecules



PsK-n

n=5, 7, 9, 11

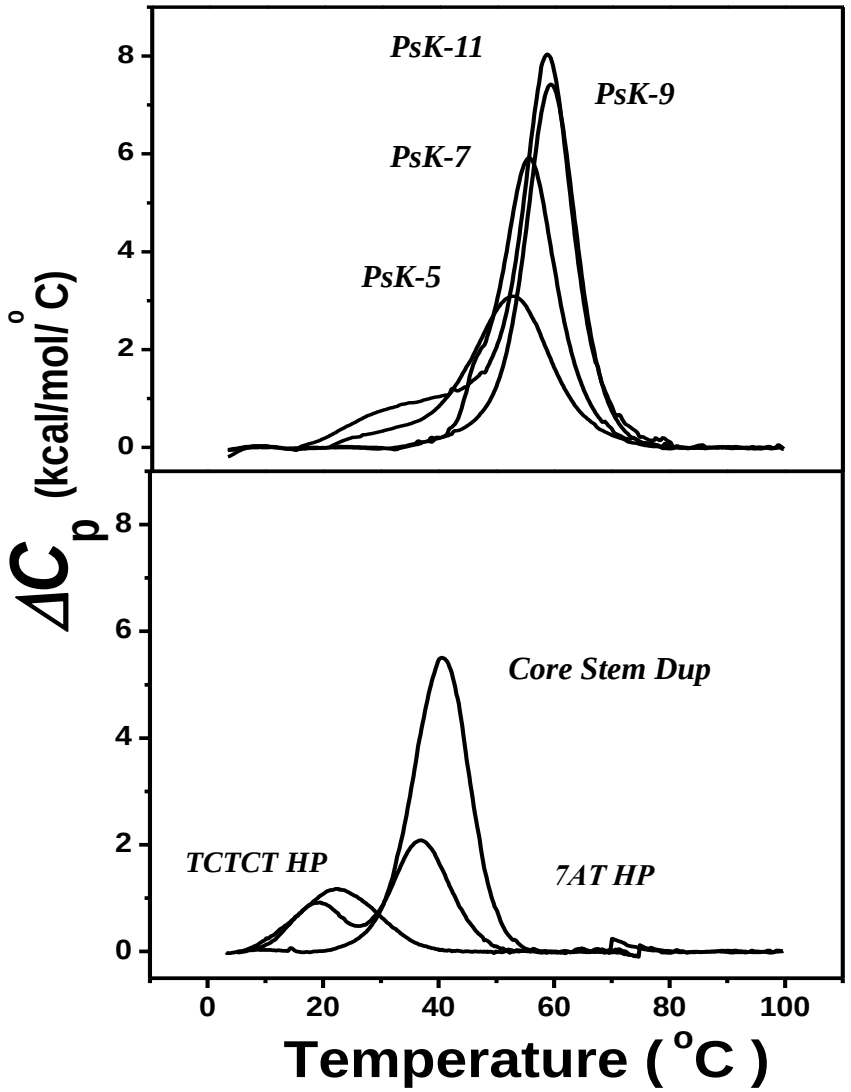
Control Molecules:

Core Dup Stem

7AT HP

TCTCT HP

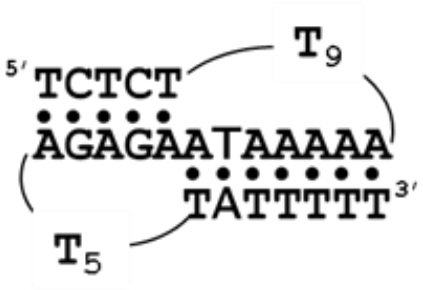
DSC Unfolding of Pseudoknots and Controls



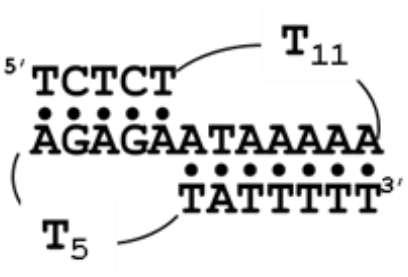
Molecule	T_M (°C)	ΔH_{cal} (kcal/mol)	$T\Delta S_{cal}$ (kcal/mol)	$\Delta G°_5$ (kcal/mol)
Pseudoknots				
PsK-5	52.8	**-60.1**	**-51.6**	**-8.5**
PsK-7	56.2	**-86.4**	**-73.2**	**-13.2**
PsK-9	59.3	**-87.5**	**-73.3**	**-14.2**
PsK-11	31.6	-26.4	-23.4	-3.0
	58.7	-83.6	-70.0	-13.6
		-110	**-93.4**	**-16.6**
Control Molecules				
Core Stem	40.5	**-69.4**	**-61.5**	**-7.9**
Dup				
7AT HP	22.8	-27.2	-12.8	-0.8
	47.6	**-35.5**	**-30.8**	**-4.7**
TCTCT HP	28.6	**-27.8**	**-25.6**	**-2.2**

- Increase in loop length yields a more favorable enthalpy
- Pseudoknot becomes more stable
- Indicates base-triplets are forming

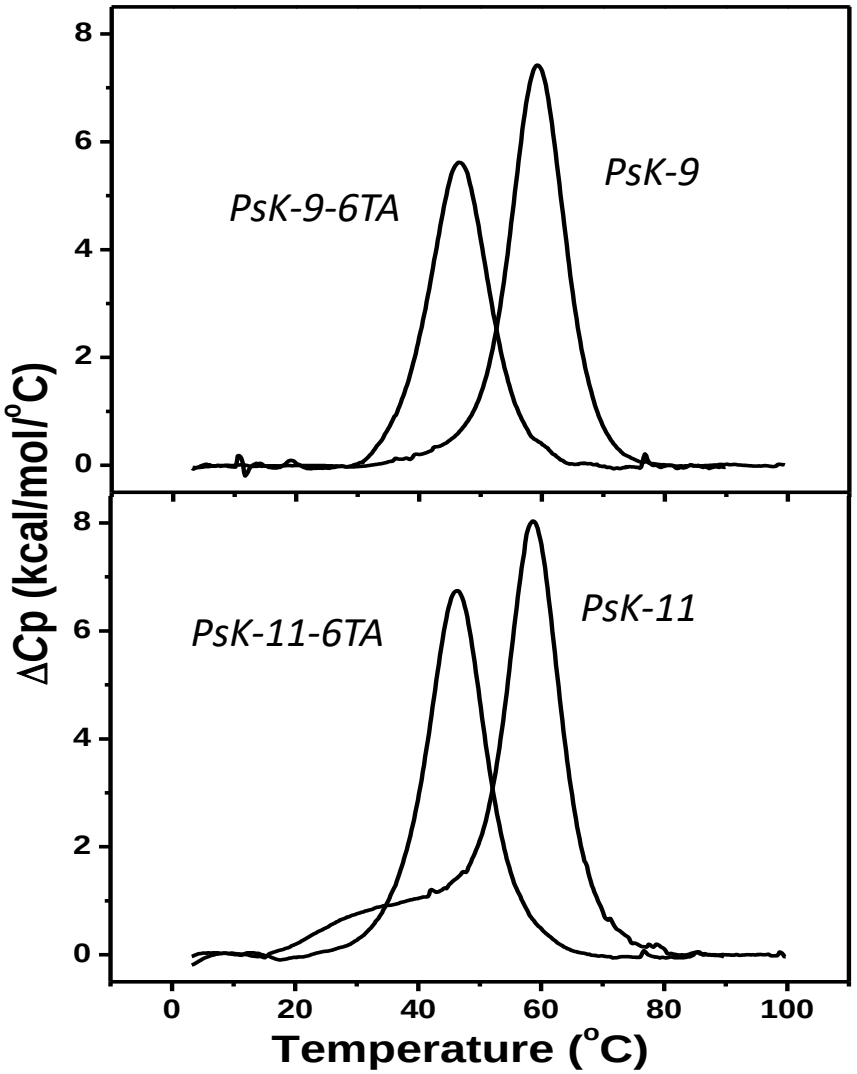
Disruption of TAT Base-Triplet Formation



PsK-9-6AT



PsK-11-6AT



Disruption of TAT Base-Triplet Formation

Molecule		T_M (°C)	ΔH_{cal} (kcal/mol)	$T\Delta S_{cal}$ (kcal/mol)	$\Delta G°_5$ (kcal/mol)
PsK-9		59.3	**-87.5**	**-73.3**	**-14.2**
PsK-9-6TA		46.6	**-72.1**	**-62.7**	**-9.4**
PsK-11	1st	31.6	-26.4	-23.4	-3.0
	2nd	58.7	-83.6	-70.0	-13.6
	Total		**-110**	**-93.4**	**-16.6**
PsK-11-6TA		46.3	**-86.2**	**-75.1**	**-11.1**

- Flipping of the AT in the core duplex disrupts the base-triplets forming in the pseudoknot with 9 thymines in the loop
  - The third strand (loop) would need to travel outside of the major groove to form a triplet with thymine now on the opposite side and then back across to continue to form. This distance is about 10Å and isn't favorable to occur

Conclusions

- DNA pseudoknots do form
- A local triplex forms in pseudoknots, provided that the appropriate loop length is complementary to the sequence of the stem.
- Each base-triplet stack increases the net enthalpy by roughly 14 kcal/mol

Take Home Message

- DSC is the only technique for directly determining the enthalpy of the unfolding of a biological polymer.
- Comparison of ΔH_{cal} to ΔH_{vH} provides unique information about the unfolding pathway (oligomerization, intermediates, aggregation).
- Sample concentration dependence of T_m is a sensitive test of higher-order association.
- Scan rate dependence of T_m is the key test for equilibrium unfolding.
- Interpretable experimental results are highly dependent on sample purity and concentration.

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- Call your local Technical or Service Representative
- Check out our Website: www.tainstruments.com

Thank You

The World Leader in Thermal Analysis,
Rheology, and Microcalorimetry