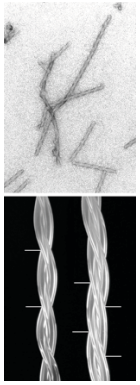
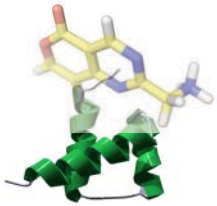
MRC | Laboratory of Molecular Biology

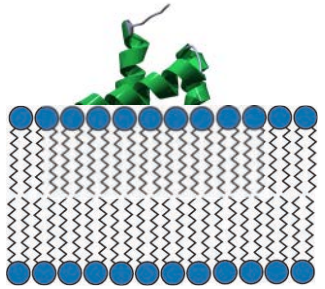
- Equilibrium Thermodynamics
- Biological Calorimetry

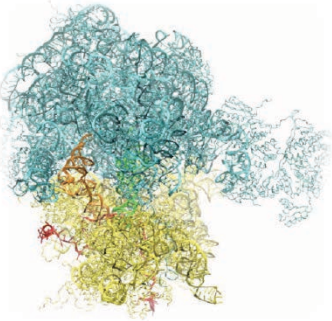
Chris Johnson
February 2020

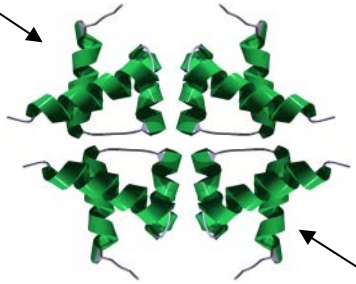
Structural and Mechanistic Molecular Biology

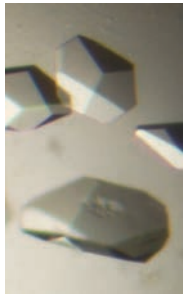










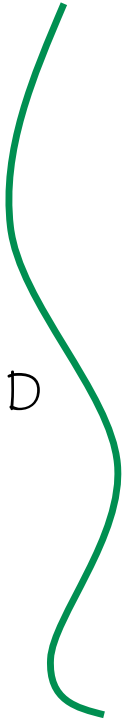


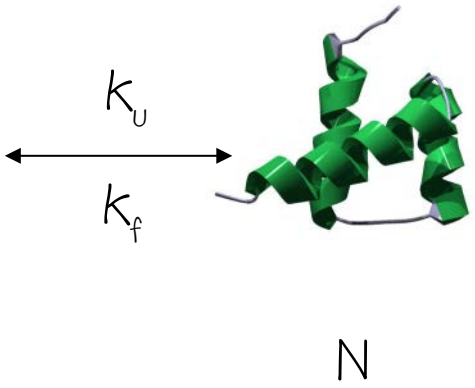
Much of biology is based around 'reversible' equilibrium processes that are mediated by consolidated non-covalent interactions which are individually 'weak' in strength

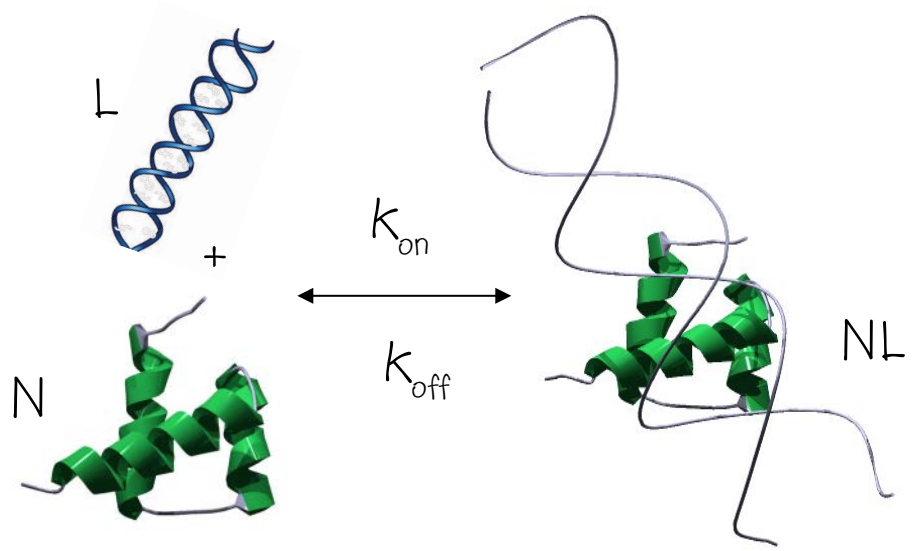
Biological Equilibrium

For example, protein folding or protein-DNA binding

- At equilibrium there is no net change in the concentration of reactants and the 'reaction' position (i.e., the biological effect) is described by the equilibrium constant, Keq, which is the ratio of the concentrations (products / reactants) which are determined by the ratio of the forward and reverse rate constants.



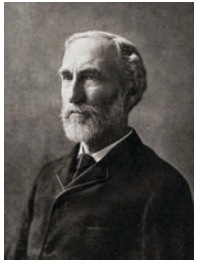




$Keq = [D]/[N] = k_u/k_f$

$Keq = Ka = 1/Kd = [NL]/[N]*[L] = k_{on}/k_{off}$

Biological Equilibrium Thermodynamics



Josiah Willard Gibbs
1839 – 1903

- The Gibbs Free Energy (ΔG) is a logarithmic representation of the equilibrium position.

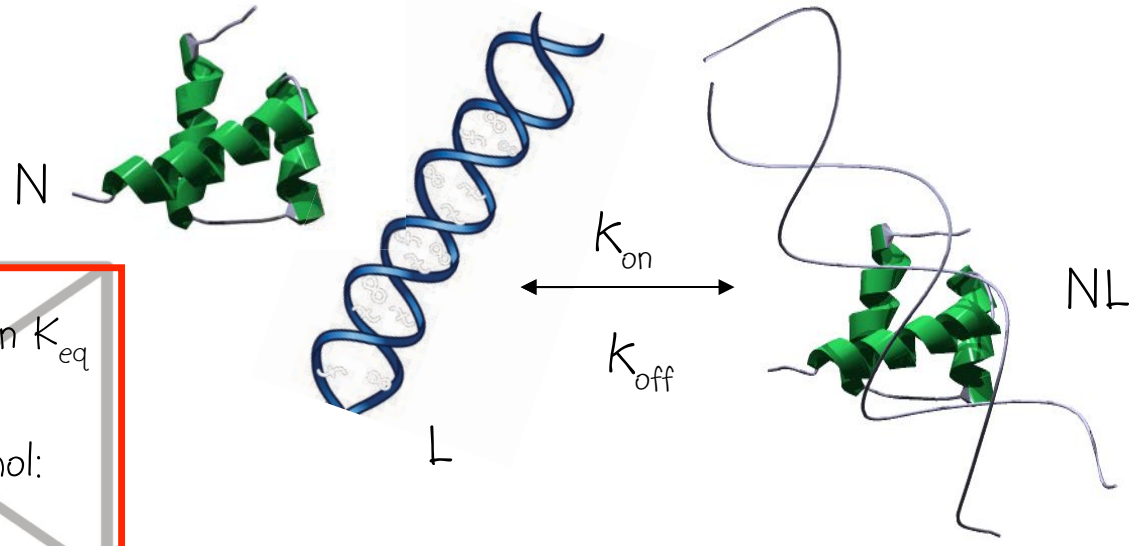
$$\Delta G = -RT \ln K_{eq}$$

- It has 'components' of 'Enthalpy' (ΔH) and 'Entropy' (ΔS)

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

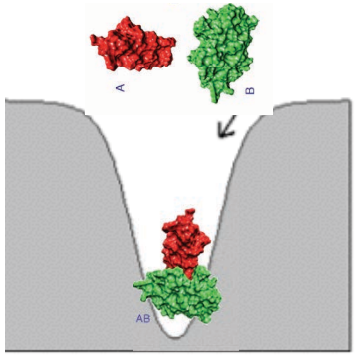
- When ΔG is -ve, the $\ln K_{eq}$ must be +ve, so the $K_{eq} > 1$ and thus <u>the products</u> are in excess (e.g., for Ka this is NL)

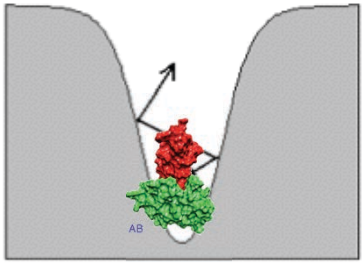
$$Ka = [NL]/[N]*[L]$$



Each order of magnitude in K_{eq} gives Δ ln 2.303
At 298K RT is 0.59 kcal/mol:
$\Delta\Delta G$ = 1.36 kcal/mol

What are Enthalpy and Entropy?
A Simplified View

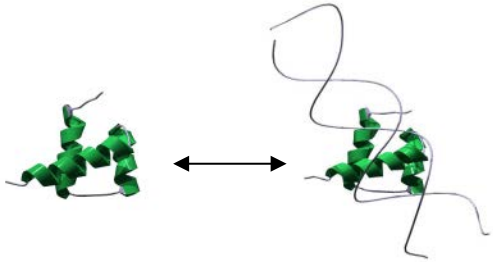




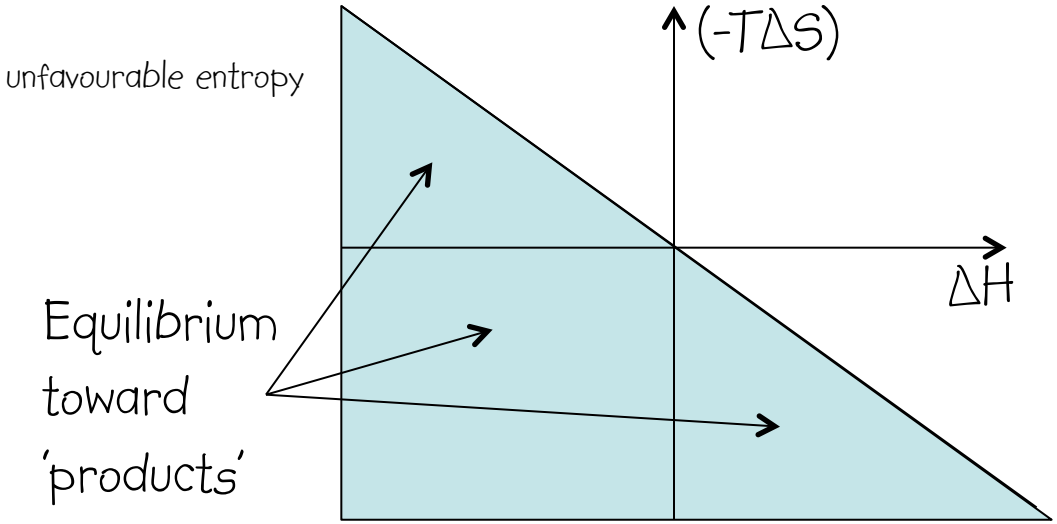
$$\Delta G = -RT \ln K_{eq} = \Delta H - T\Delta S$$

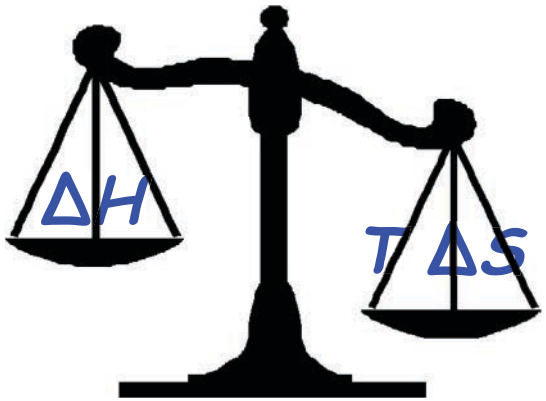
- Systems naturally progress to a lower internal energy level; lower enthalpy,
- The change, ΔH, is -ve and heat given off, "exothermic", equilibrium product favoured.
- Systems want more ways of configuring with the same energy (more 'disorder'). Statistically the more ways of achieving an outcome there are the more probable that outcome. Temperature modulates this probability effect through thermal motion ($-T\Delta S$)
- Entropy is a measure of the number of ways of arranging system energy; ΔS, is +ve (more ways) equilibrium product favoured ($-T\Delta S$ is -ve)

Where does the equilibrium end up?



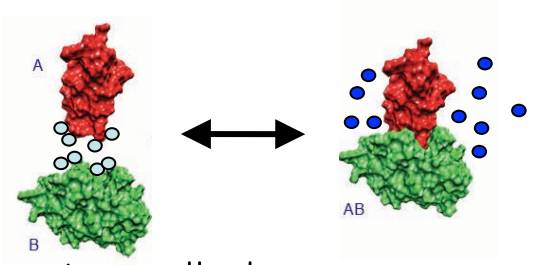
- Simply the balance of enthalpy and temperature-entropy gives ΔG, and thus defines the equilibrium position.
- Temperature is a key factor through TΔS term
- A -ve ΔG is 'favourable' and can be obtained even when one component is 'unfavourable' if the other is 'favourable' and larger





Are ΔH and ΔS Fixed values?

Changes in heat capacity



- In biological equilibria there are changes in structure and usually large changes in solvation both of which which affects the system heat capacity (Cp), that is the energy needed to increase temperature by 1 °K, units, cal mol^{-1} K^{-1}. These reactions have a **change** in heat capacity: ΔCp
- The integral of Cp or Cp/T from zero K to temperature T represents the enthalpy and entropy levels for a system
- Because of the significant ΔCp for biological equilibria the corresponding ΔH, ΔS (and thus ΔG) are all temperature dependent with ΔS in a non linear manner

$$\Delta G_{T_2} = \left[\Delta H_{T_1} + \Delta Cp.(T_2 - T_1)\right] - T_2\left[\Delta S_{T_1} + \Delta Cp.\ln\left(\frac{T_2}{T_1}\right)\right]$$

Other than temperature and ΔC_p can anything else affect ΔG?

Henry Louis Le Chatelier
1850-1936

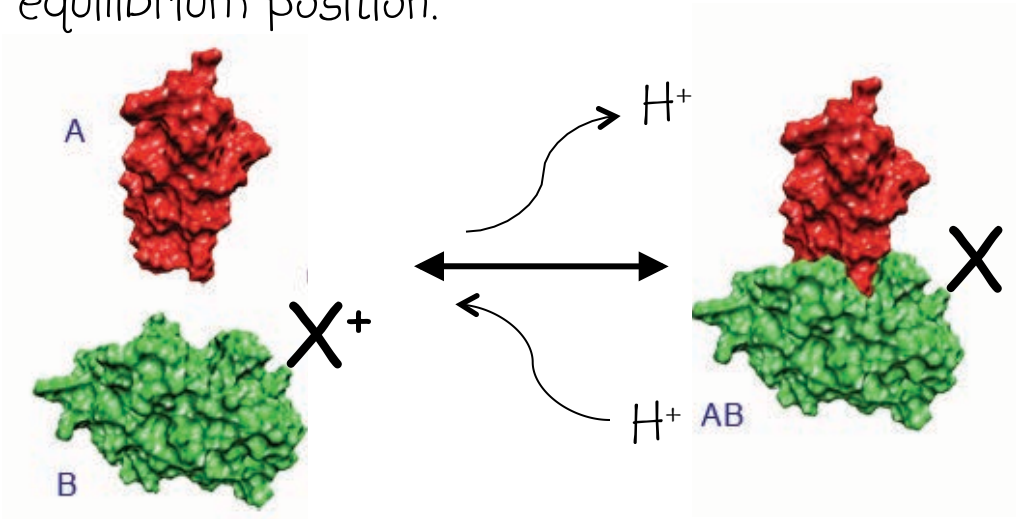
... many things because ....

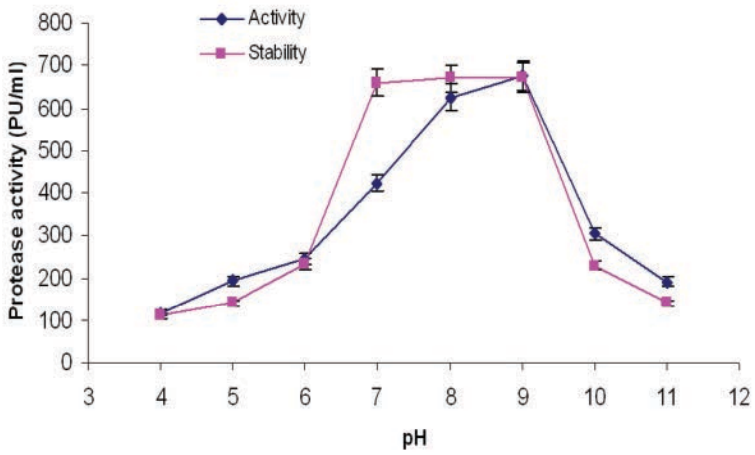
Law of Mass Action; Le Chatelier's principle

"When a system at dynamic equilibrium is disturbed, the equilibrium position will shift in the direction which tends to minimise, or counteract, the effect of the disturbance"

Protein-protein Interaction with a change in protonation

- The pKa's of groups change on complex formation due to different envirnonment or specific electrostatic interactions
- Thus the equilibrium position changes the proton concentration (although pH change is prevented by the buffer)
- Therefore (mass action) proton concentration (pH) will affect the equilibrium position (ΔG)
- If there are no pKa shifts in an equilibrium, there is no pH dependence in the equilibrium position.

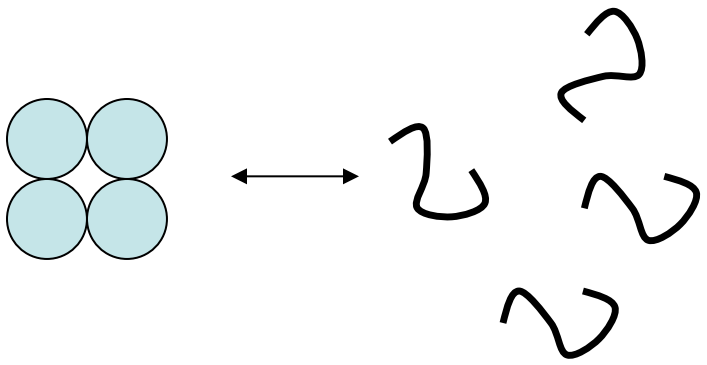


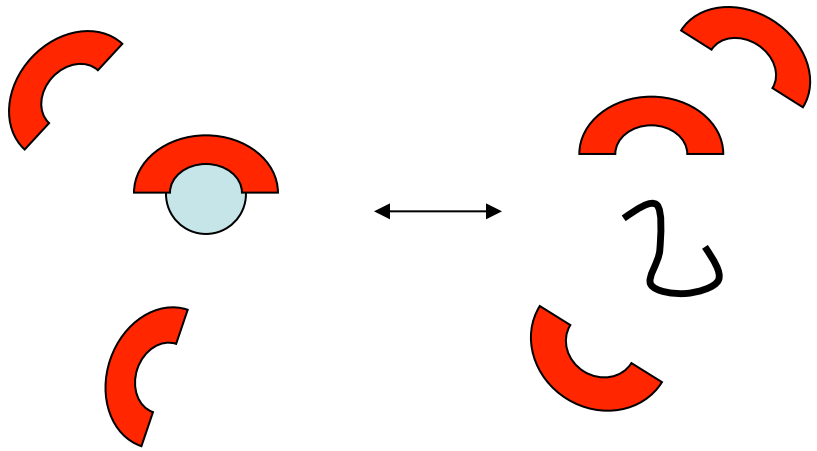


Other Examples of Mass Action....

- Equilibrium changes concentration of molecules
- Therefore, equilibrium (stability, melting temperature) depends on the concentration

- Equilibrium involves changes in binding of ligand
- Therefore, the equilibrium (chemical stability, melting temperature) depends on concentration of the ligand

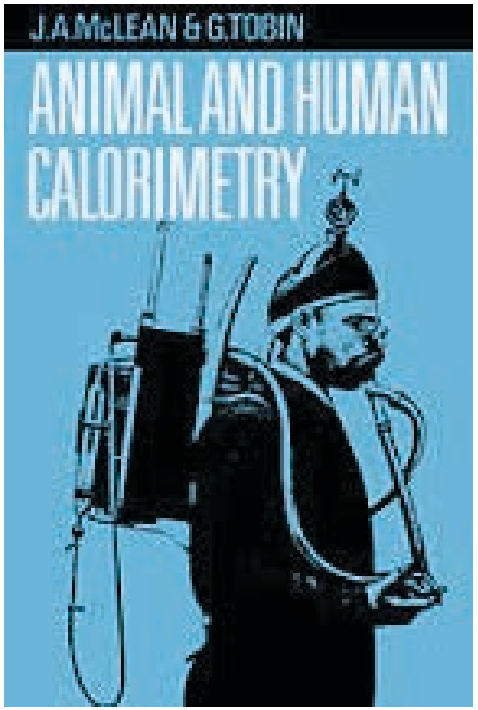


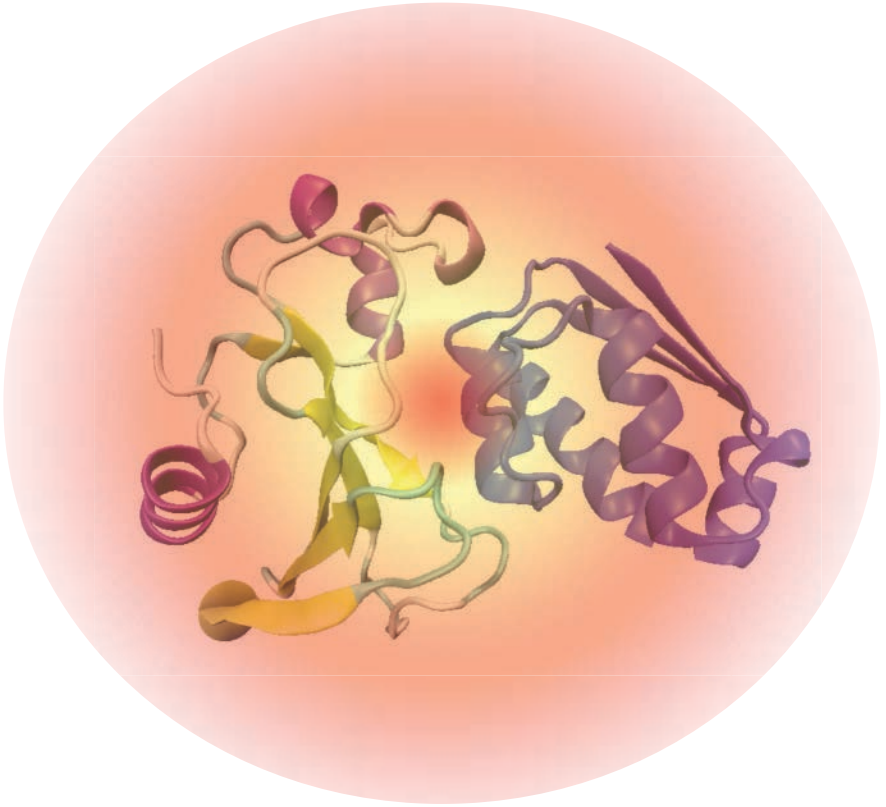


Biological Calorimetry

How to measure Enthalpy?

Under constant pressure the **heat** transferred during a process is the enthalpy (ΔH).
Calorimetry (Calor; heat Metrum; measure) is thus a technique that measures directly enthalpies of processes.





Calorimetry might be useful then?

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

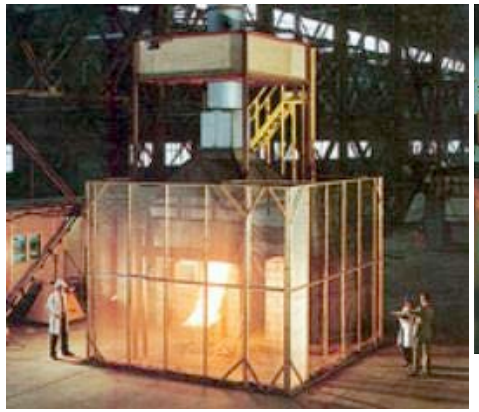
$$\Delta G = -RT \ln K_{eq}$$

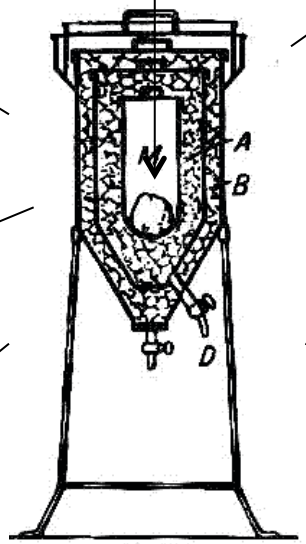
- The signal in a calorimeter is the 'heat' of a process, the change in enthalpy (ΔH), which is a direct measurement of one of the driving forces of biological equilibria

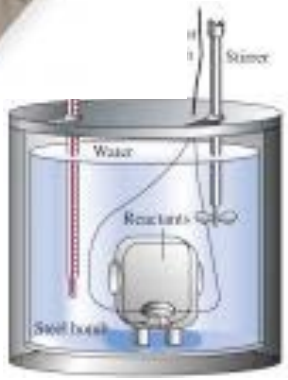
Also calorimetry is .....

- Able to determine the value of $T\Delta S$ (if we measure ΔG (K_{eq}) and ΔH)
- A very general method. All biological equilibria have an associated enthalpy (conformational transitions, melting, binding/interaction, turnover/catalysis, etc.). No method or assay development is required.
- A non-optical, label free method. No specific group(s) or label(s) required, can use turbid suspensions or crude extracts, unusual solvents, high backgrounds of other molecules, etc. Thus applicable to many systems from the molecular to cellular level (so long as material fits in the instrument !)

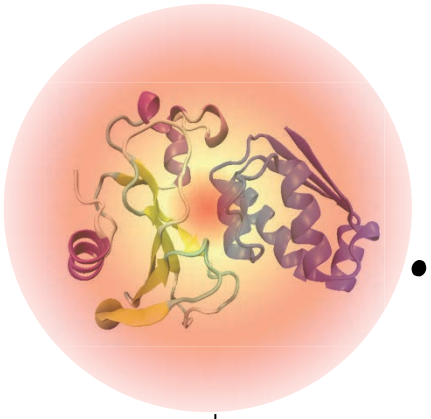
Calorimetry was one of the earliest techniques reported in the 'literature' and is now used in many areas

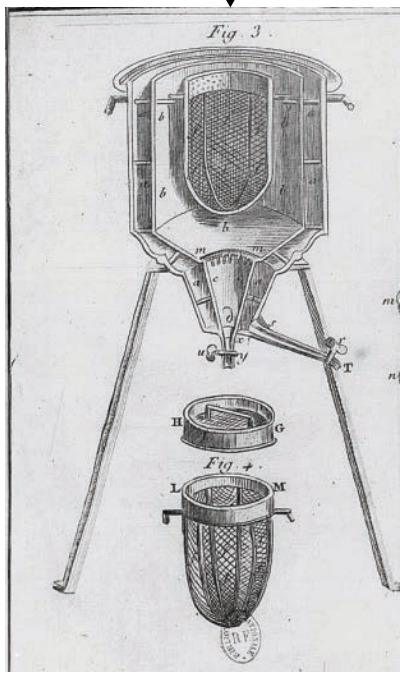






- Lavoisier's ice calorimeter 1780's
- Respiration is 'combustion'





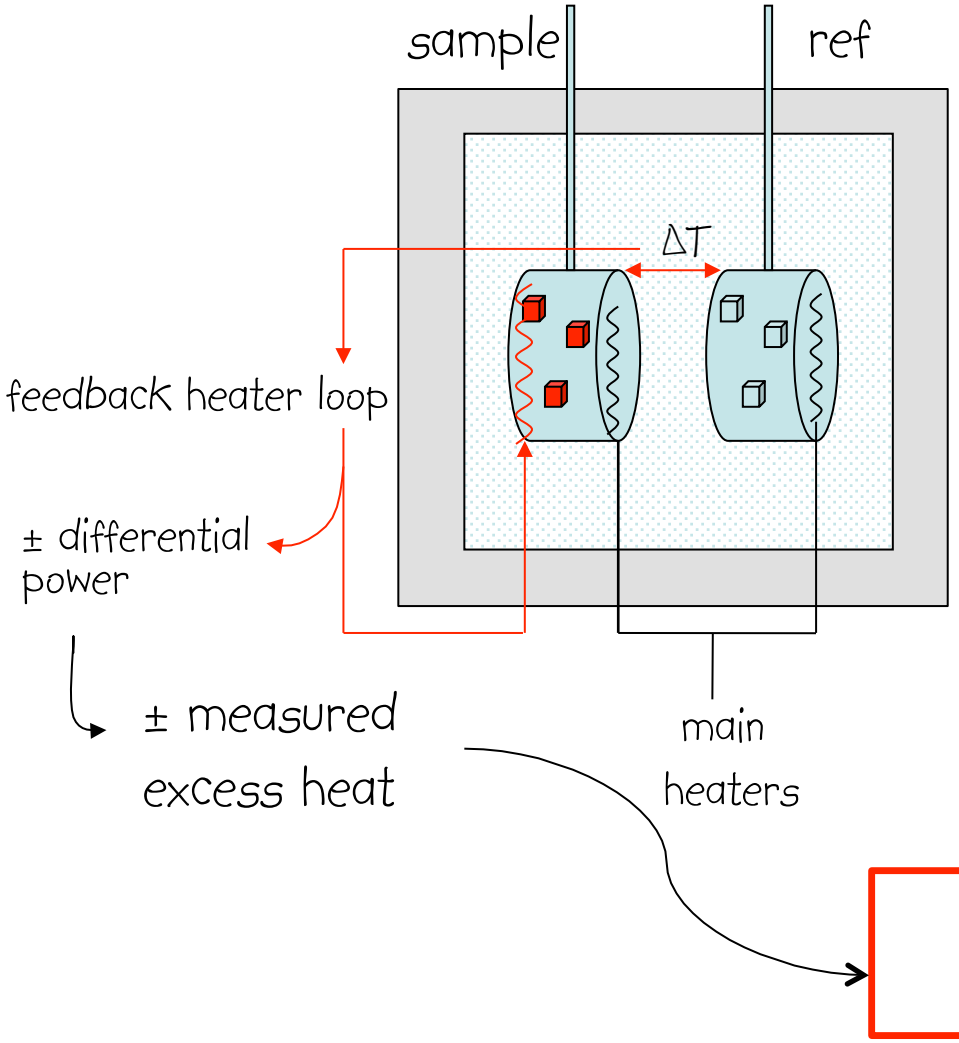
'Biolcalorimetry'

- Heat (enthalpy, ΔH) from a typical protein-protein interaction might be -10 kcal mol^{-1} *.
- Thus, 50 nmol (1ml of 50 μM solution) will give off -5 x 10^{-4} cal heat upon binding thus melting 6 μg ice or heating 1 ml water by 5 x 10^{-4} °C.
- Conventional direct heat transfer calorimetry is not possible.

Antoine-Laurent de Lavoisier
1743-1794

*Kd 50nM @ 298K ΔS =0

Power Compensation Biocalorimeters



- Difference in temperature relative to an 'identical' reference cell containing solvent measured by very precise thermopile and is kept constant by the calorimeter in a feedback loop controlling electrical heating to the sample cell.
- Increases or decreases in differential power in this circuit are directly proportional to the excess heat taken up or given off during 'reactions'

Types of Biocalorimetry

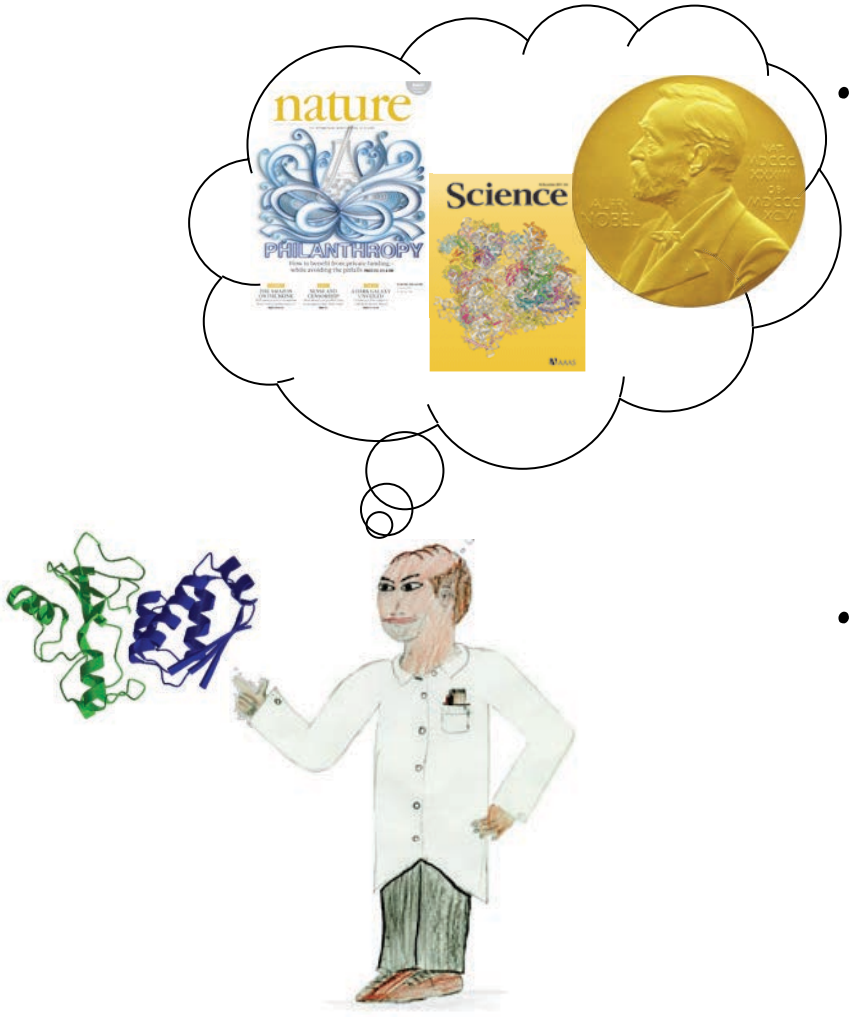
- Isothermal Titration Calorimeters (**ITC**); study interactions through the titration and mixing of two molecules at constant temperature (4-70°C : Kd; mM to nM)

- Differential Scanning Calorimeters (**DSC**); study temperature-induced transitions, melting, by increasing or decreasing temperature (Tm; 0-130°C)

Int J Pept Protein Res. 1973;5(4):229-37.
Calorimetric investigation of ribonuclease thermal denaturation.

Anal Biochem. 1989 May 15;179(1):131-7.
Rapid measurement of binding constants and heats of binding using a new titration calorimeter.

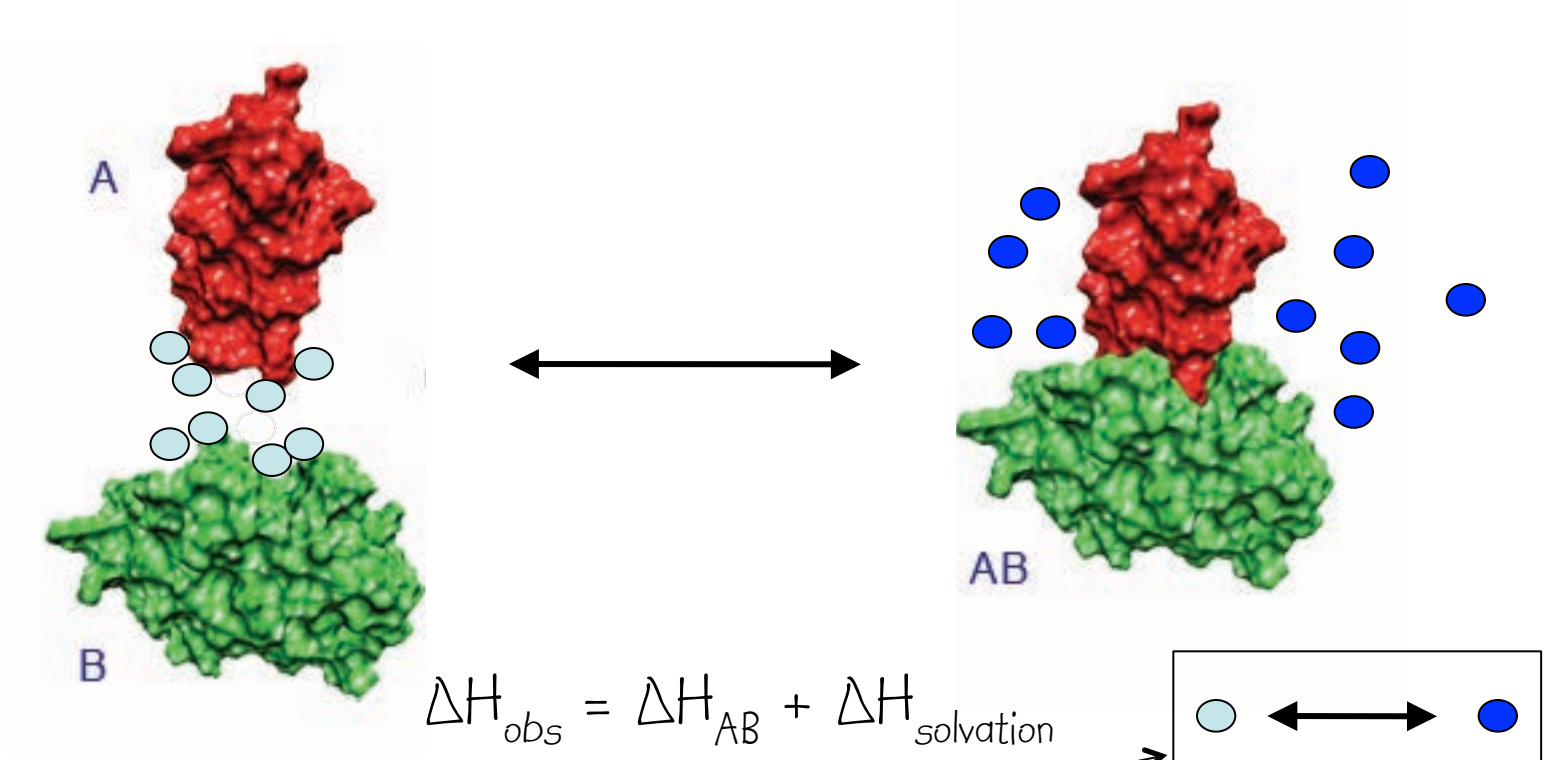
I can see interactions in my structure and mutate them so I can measure and manipulate their energies directly?!!



- Measured heat (Enthalpy) is a global non-specific probe and calorimetry measures the totality of heat effects from **all changes** in a system, i.e., specific structural interactions (observable by cryoEM, X-ray or NMR), but also changes in solvation, shifts in pKa's (i.e., changes in protonation), changes in dynamics, etc.
- This emphasises the combination of molecular forces driving the interaction process in solution (because these are not resolved in structures they are often forgotten or ignored).

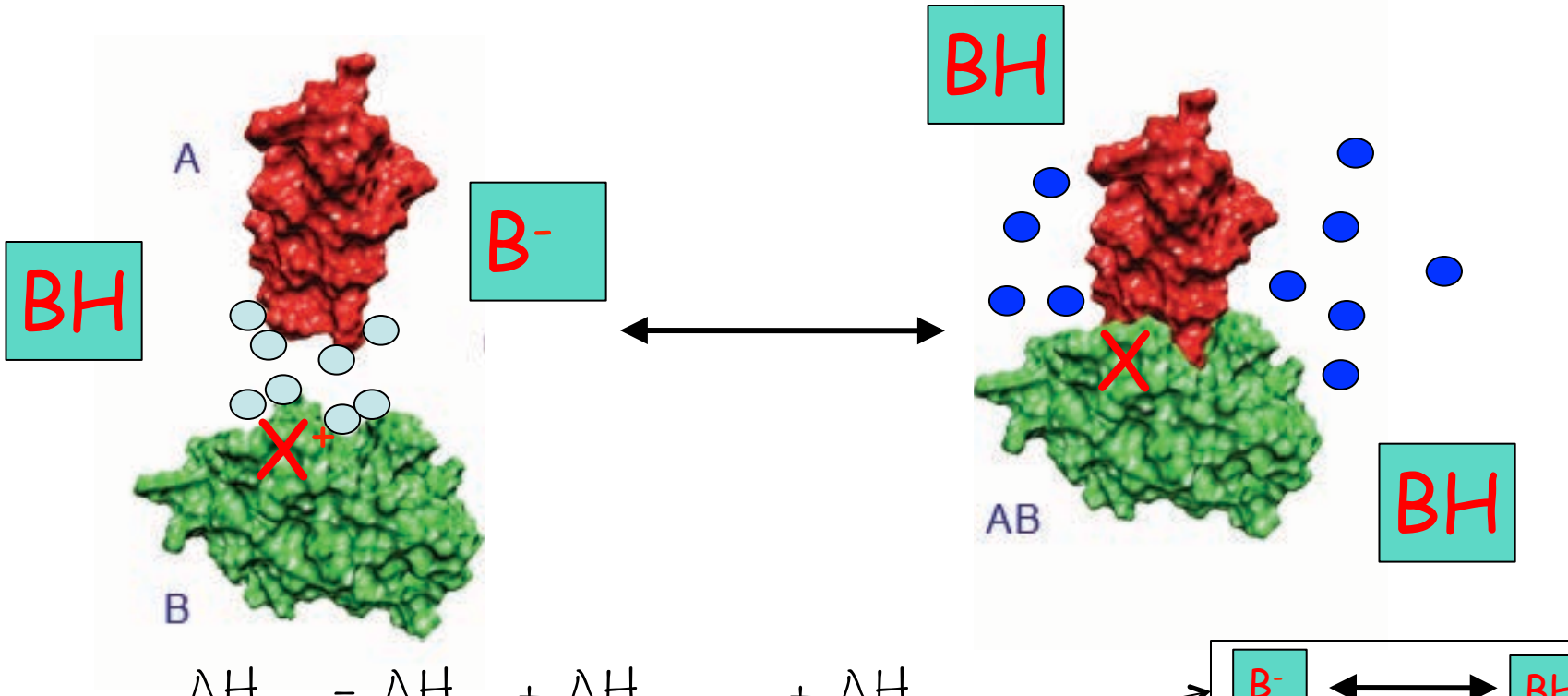
Interactions in Solution

- Interactions in solution involve changes in solvation and the observed thermodynamics are the sum of all contributions
- Proteins only interact *in vacuo* in the figures of journals !



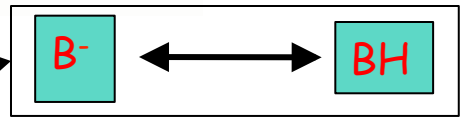
Interactions in Buffers

- Buffers take up or donate protons during the interaction and this also has an associated enthalpy

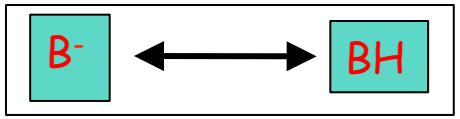


$$\Delta H_{obs} = \Delta H_{AB} + \Delta H_{solvation} + \Delta H_{ionisation}$$

$$\Delta S_{obs} = \Delta S_{AB} + \Delta S_{solvation} + \Delta S_{ionisation}$$



Calorimetry can measure directly net protonation changes (Δv)



- Δv is the sum of all protonation changes involved in the reaction

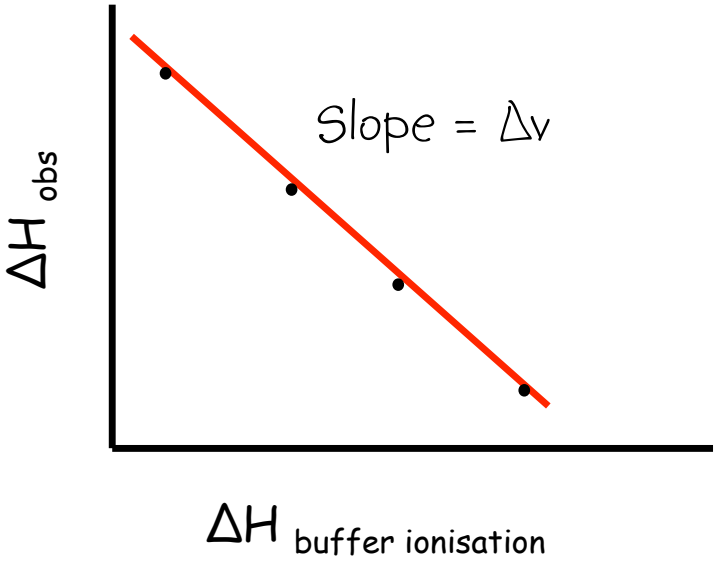


TABLE I. Entalpy and Heat Capacity Changes for the Dissociation of Protonated Buffer Substances in 0.1 M KCl at 25° C

Buffer substances	p*K**	ΔH (kJ mol^{-1})	ΔC_p (J K^{-1} mol^{-1})	$\partial\Delta C_p/\partial T$ (10^{-3} J K^{-2} mol^{-1})
Acetate	4.62	0.49 ± 0.02	−128 ± 2	—
MES	6.07	15.53 ± 0.03	16 ± 2	—
Cacodylate	6.14	−1.96 ± 0.02	−78 ± 2	—
Glycerol 2-phosphate	6.26	−0.72 ± 0.02	−179 ± 2	0.79 ± 0.39
PIPES	6.71	11.45 ± 0.04	19 ± 4	—
ACES	6.75	31.41 ± 0.05	−27 ± 4	—
Phosphate	6.81	5.12 ± 0.03	−187 ± 3	2.01 ± 0.22
BES	7.06	25.17 ± 0.07	2 ± 5	—
MOPS	7.09	21.82 ± 0.03	39 ± 3	—
Imidazole	7.09	36.59 ± 0.06	−16 ± 5	—
TES	7.42	32.74 ± 0.03	−33 ± 3	—
HEPES	7.45	21.01 ± 0.07	49 ± 5	—
EPPS	7.87	21.55 ± 0.05	56 ± 4	—
Triethaolamine	7.88	33.59 ± 0.04	48 ± 3	—
Tricine	8.00	31.97 ± 0.05	−45 ± 4	—
Bicine	8.22	27.05 ± 0.05	2 ± 4	—
TAPS	8.38	41.49 ± 0.06	23 ± 5	—
CAPS	10.39	48.54 ± 0.07	29 ± 6	—

*Standard error is within ±0.01.

Fukada and Takahashi (1998)
PROTEINS: Structure, Function, and Genetics
33,159

Practical Biocalorimetry

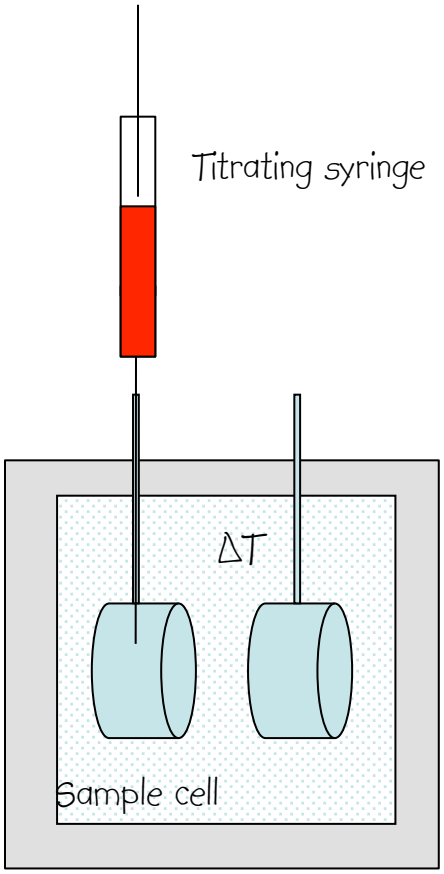
Types of Biocalorimeter

- Isothermal Titration Calorimeters (ITC); study interactions through the titration and mixing of two molecules at constant temperature (4-70°C : Kd; mM to nM)

- Differential Scanning Calorimeters (DSC); study temperature-induced transitions, "melting", by increasing or decreasing temperature (Tm; 0-130°C)

ITC

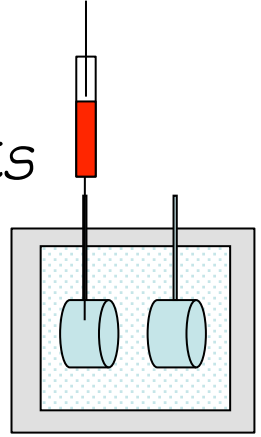
Typical ITC Experiment



- iTC 200 instrument (active cell volume 200 uL, syringe 40uL)
- 350 uL of 'target' 10 - 50uM* loading cell (275 uL consumed)
- 70 uL of 'ligand' 50 – 500 uM* loading syringe (55 uL consumed). Make 15-20 injections 2-3uL
- ITC is a 'sample hungry' technique so evidence of binding from other techniques and/or the literature is useful.
- Heat is a non-specific probe and calorimetry measures the totality of heat effects from all events .... Controls needed!

* Typical starting concentrations. Actual concentrations depend on ΔH (signal amplitude), Kd of binding and the type of experiment

Sources of signal and controls for ITC experiments



① Buffer injected into buffer. The background heat of 'injection'. Mechanical disturbances and temperature gradients. ○

② Buffer injected into protein. Background heat plus the heat of dilution of the protein in the cell. Usually = background as dilution factor in the cell is small. ○ + ○

③ Ligand injected into buffer. Background heat plus heat of dilution of the ligand. Significant! Dilution of ligand is large ○ + ●

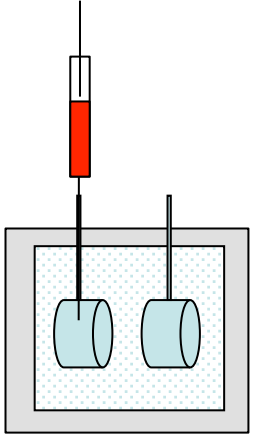
④ Ligand injected into protein. Background plus heat of dilution of protein and ligand with heat of binding. ○ + ○ + ● + ●

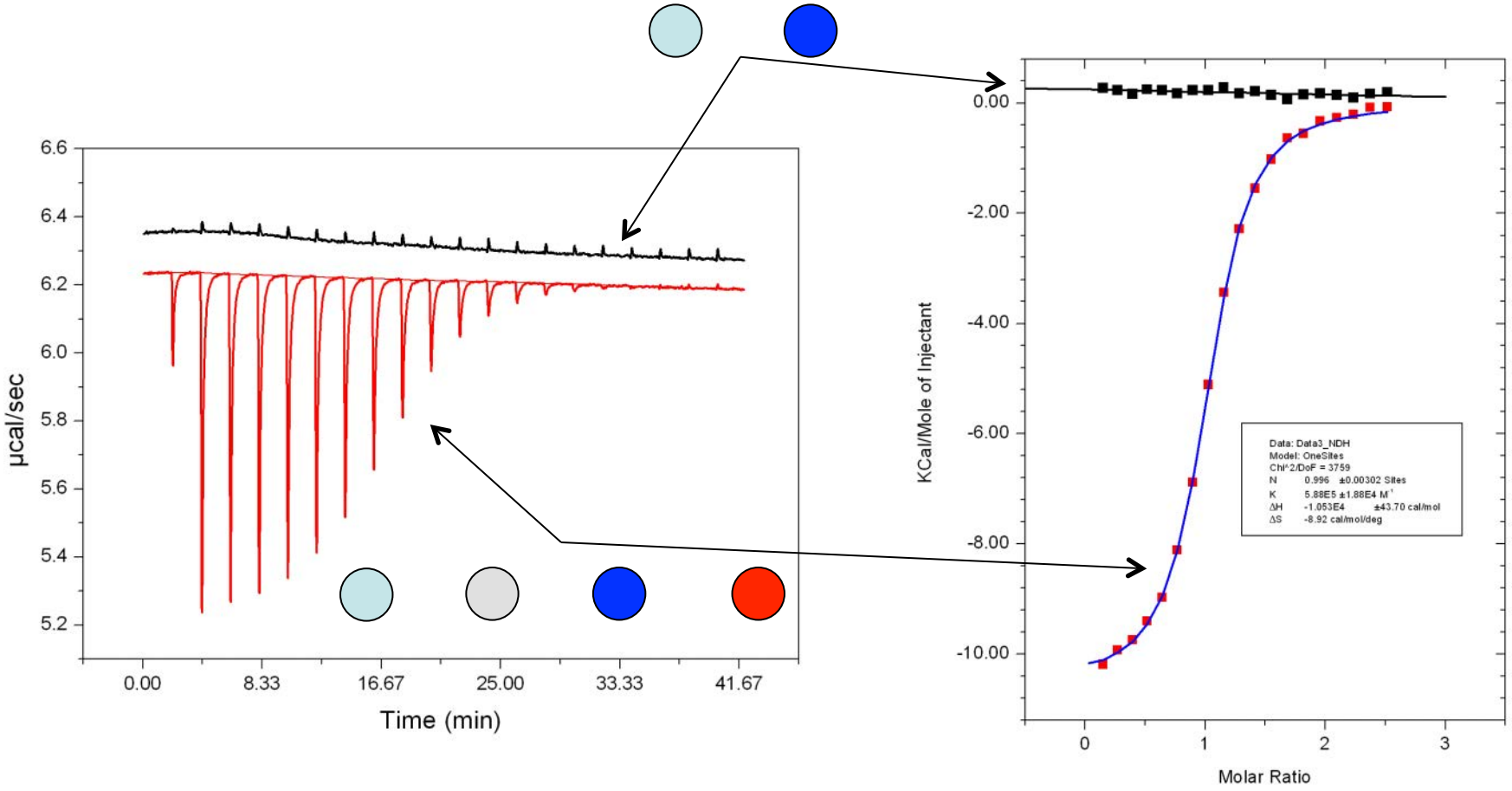
Since ○ is ~ 0 then 4 should be corrected with 3 to give ●

Example: ITC measurement and separate control

600uM peptide into 45 uM protein or into buffer

Integrate heat from each injection. Subtract control and fit.

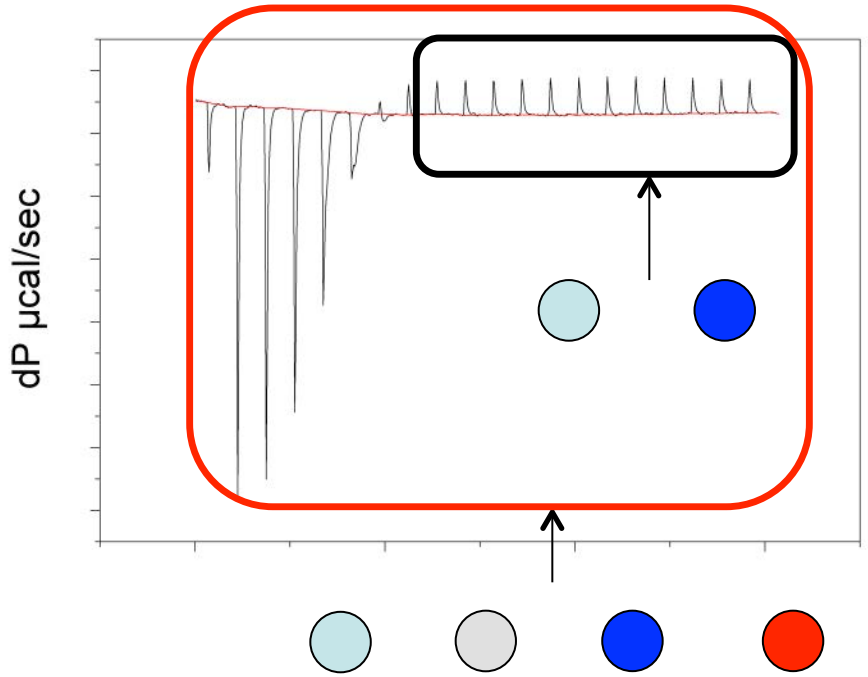


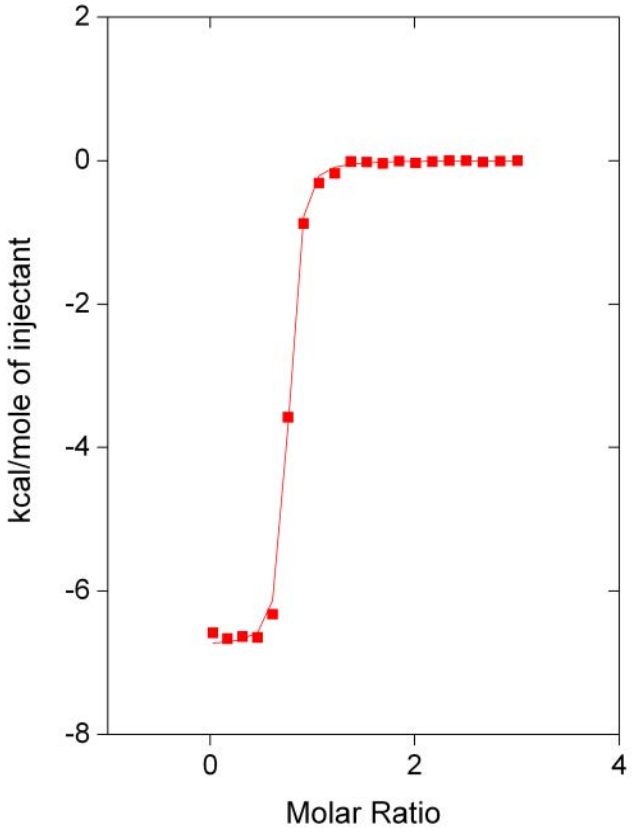


Single experiment measurement

500uM ligand into 30uM protein

Integrate heat from each injection. Subtract 'control' heats observed at 'saturation' toward the end of the titration





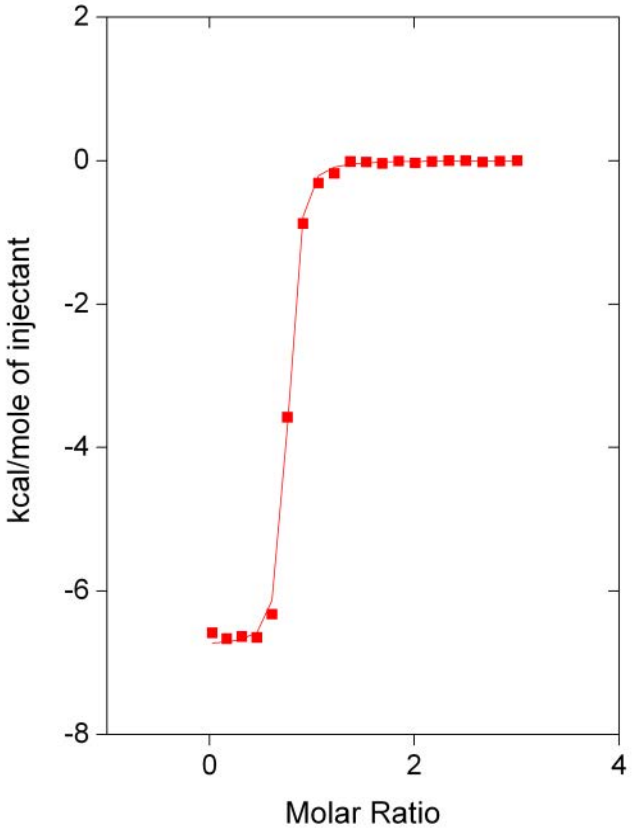
Data Fitting in Origin or PEAQ*

Simple model of N site(s) with identical affinity and enthalpy

N	0.703	0.00294
Ka	2.98E6	3.61E5 M-1
Kd	330 nM	+/- 40 nM
ΔH	-6759	41.60 cal/mol

Calculated values

ΔG	9 kcal/mol
ΔS	7 cal/mol/deg

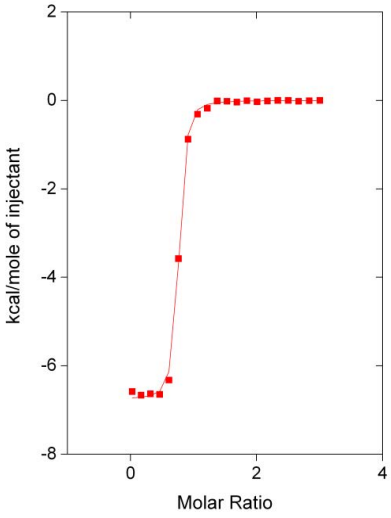


- Disregard the errors and the suggested precision
- Plot in conventional format and refit
- Come to talk 16

*Software provided with Microcal calorimeters

The 'meaning' of N = 0.7

- 'N' is the 'number of sites', or 'stoichiometry' of the interaction and is well determined for this tight binding
- It assumes the concentration of the macromolecule in the cell and the ligand in the syringe are measured correctly and both are 100 % 'pure' and 100% 'native' or 'binding competent'.
- 'N' can therefore be viewed as an indicator of 'active site concentration' or an additional fitting parameter that allows concentration to float during fitting and thereby allow the fit to go through the points.
- In practice N = 1 ± 0.2 is equivalent to N ~ 1. N = 0.5 and N = 2 etc., also have some potential physical meaning (e.g., dimer with one site etc, etc)

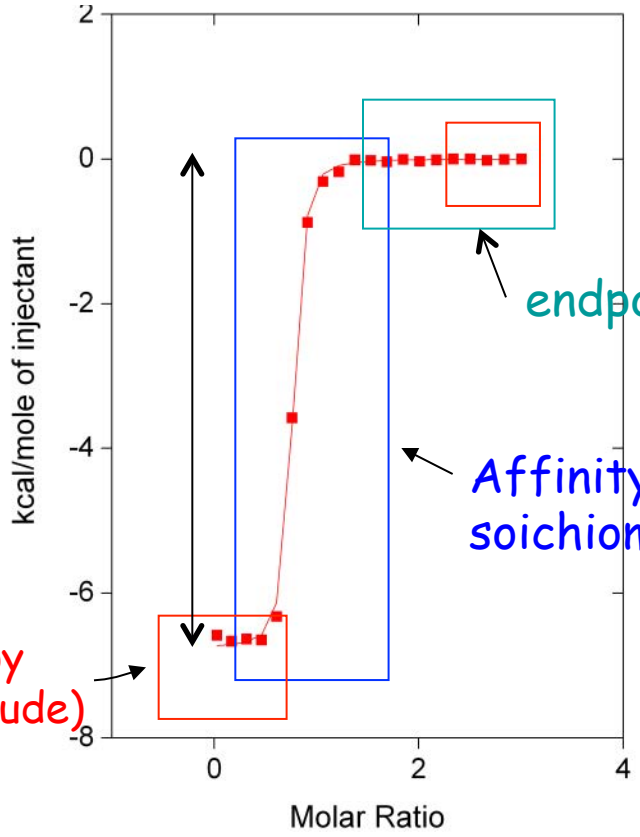


NLLS Fitting values	
N	0.703
Ka	2.98E6
Kd	330 nM
ΔH	-6759

Information content and optimising ITC experiments

Derivative plots can be described by parameter known as 'C value' = [protein cell] / Kd

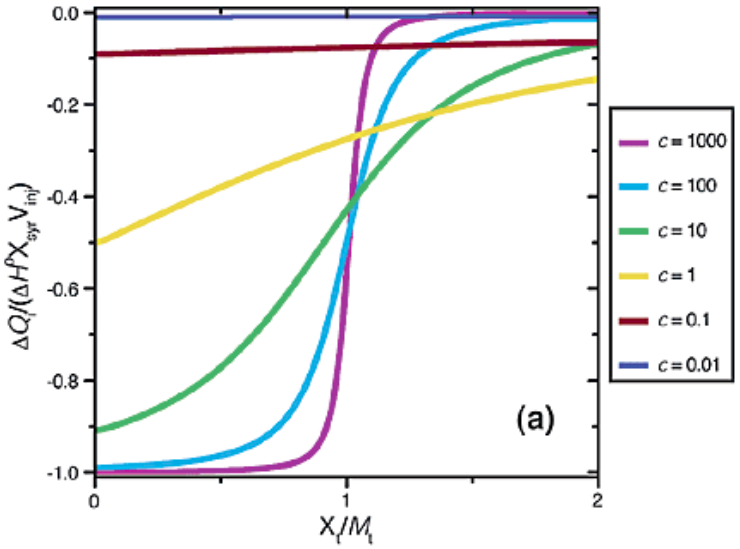
Optimal range for ITC 10 < c < 500 when trying to determine Kd, N and ΔH in one experiment



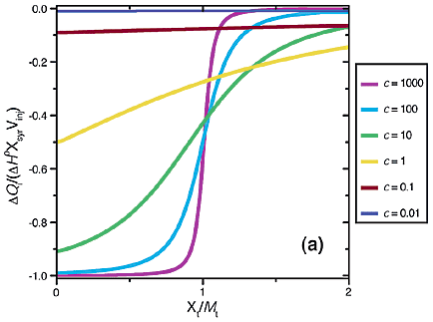
endpoint

Affinity and soichiometry

Enthalpy (Amplitude)



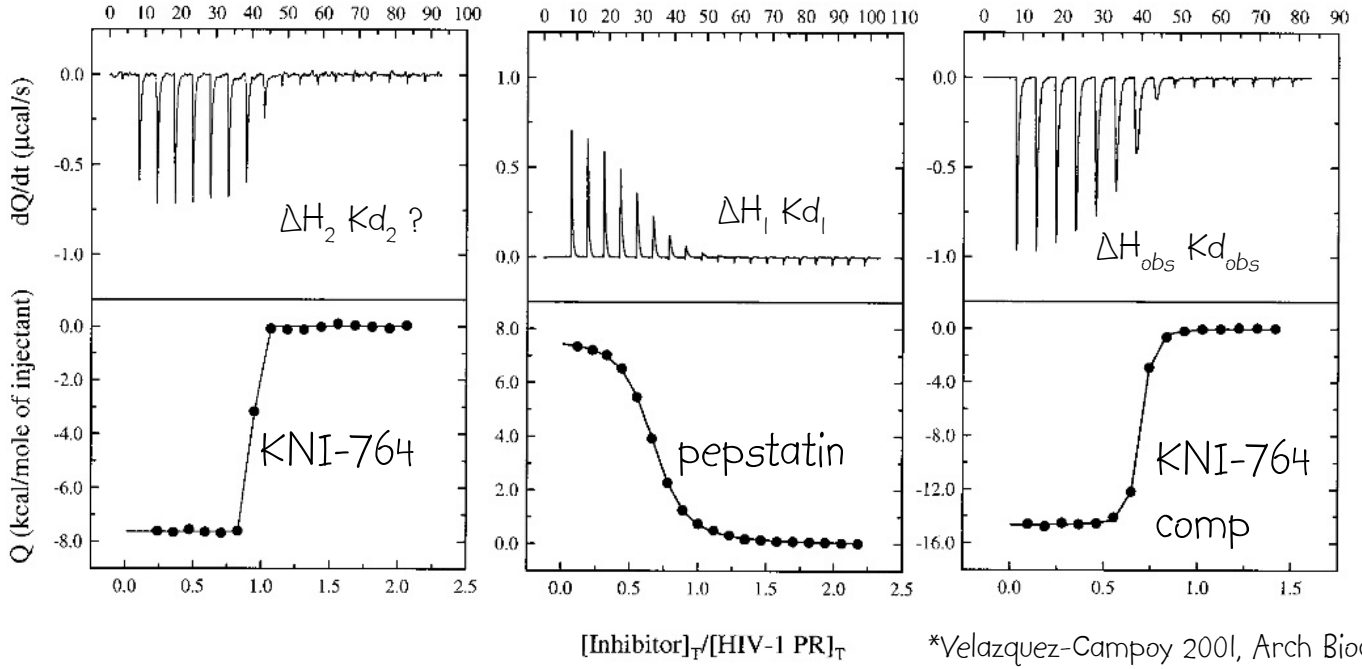
High affinity binding



- As binding becomes tighter c-value ([cell]/Kd) becomes too large unless protein concentration is decreased.
- However, (cell concentration x ΔH) determines the total heat in the experiment and with current detection limits [protein] must be > 5-10 uM unless ΔH is very large.
- This limits simple ITC measurements to Kd's in the 100's nM range
- However, since ITC is a non-optical and probe free method it is perfect for displacement (competition) binding methods which can extend the Kd range down to pM and beyond.

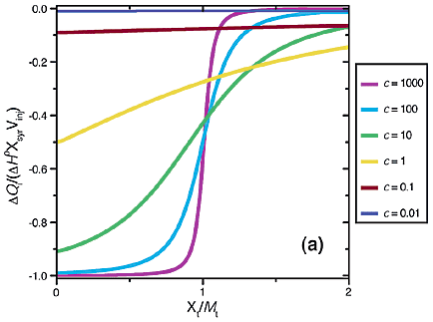
Competition binding experiments

- Measure first a 'weaker' binding ligand (pepstatin). Displace this ligand in a second titration of the endpoint complex with the tight binder (KNI-764)
- ΔH_{obs} competition ~ = ΔH_2 + (- ΔH_1)
- Kd_{obs} competition ~ = Kd_1 x Kd_2 KNI-764 inhibitor binding HIV-1 protease Kd 32 pM
- Note. Both affinity and enthalpy change making this an ideal tool for screening small molecules, for example, that compete for a target binding site



*Velazquez-Campoy 2001, Arch Biochem Biophys, 390, p169
Velazquez-Campoy 2006 Nat Protoc;1(1):186-91

Weak binding



- As binding becomes weaker, the c-value ([cell]/Kd) becomes small unless [protein] is increased. Kd, ΔH and n are all less well constrained and it may be difficult to define titration endpoints.
- The background heat of dilution of the ligand (which may be required at 10's mM concentration in the syringe) is critical and so separate titrations of ligand into buffer are required.
- Many small molecule fragments require 5-10% DMSO to dissolve at mM concentrations. This exacerbates the background heat of dilution if DMSO is not carefully matched in cell and syringe.
- If material and solubility are not limiting, very weak binding or non-specific interactions can be quantified

- Turnball et al., J Am Chem Soc. 2003, 125(48):14859-66
- Tellinghuisen. Anal Biochem. 2008; 373(2):395-7

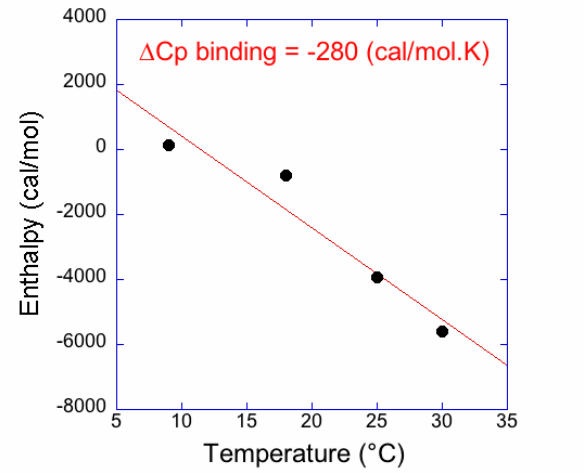
No just Kd's and ΔH

ΔCp of binding

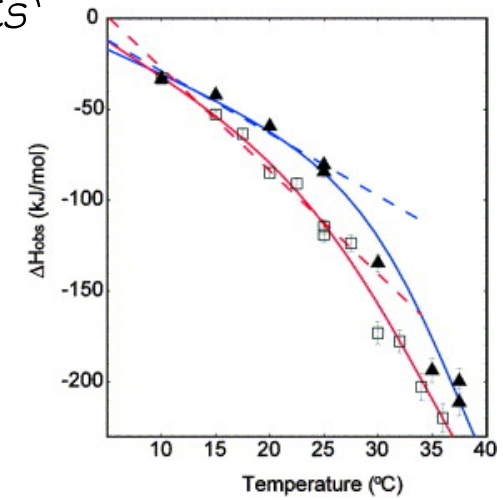
$$\Delta H = \int_0^T \Delta Cp . \partial T$$

$$\Delta Cp = \frac{\partial \Delta H}{\partial T}$$

- ΔCp can be obtained from the variation of ΔH with temperature.
- Rigid body type interactions have a constant typically -ve ΔCp^{bind}
- Unlike ΔH, ΔCp^{bind} can be deconvolved to indicate properties of the ligand binding footprint, (ΔASA, non polar, polar etc.)



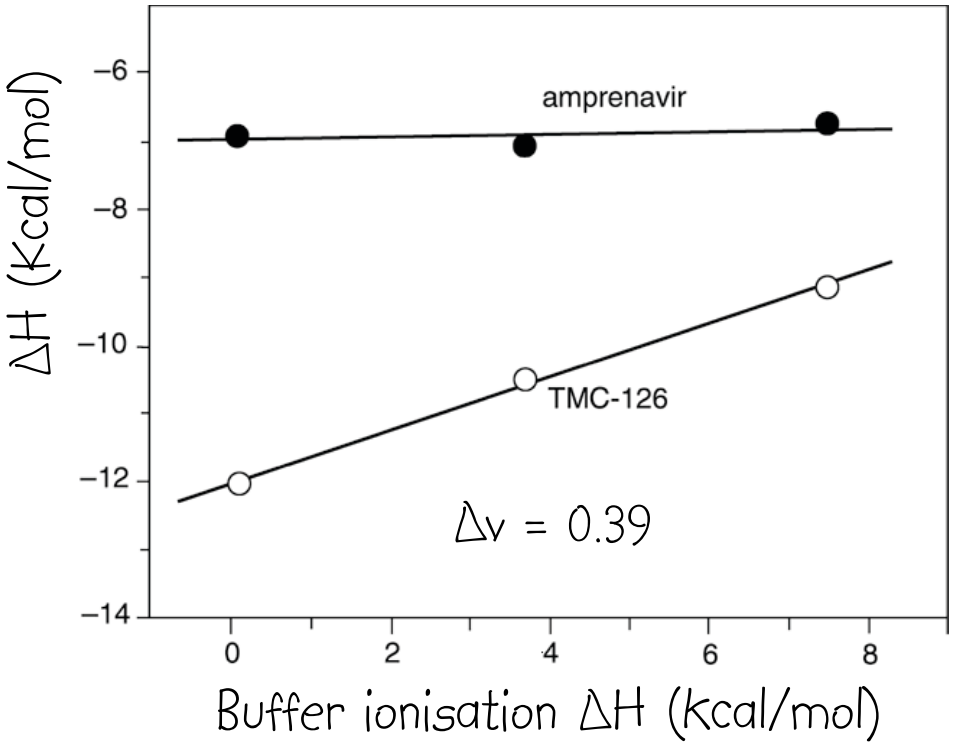
- Non-constant or large values for ΔCp^{bind} indicate coupled events such as ligand induced structuring (NDP folding and binding) or conformational change (domain movements)



- Molecular recognition via coupled folding and binding in a TPR domain. Cliff MJ et al, J Mol Biol. 2005 346:717-32
- Vega et al., Biochim Biophys Acta. 2016;1860(5):868-878

Δv (protonation change) of binding

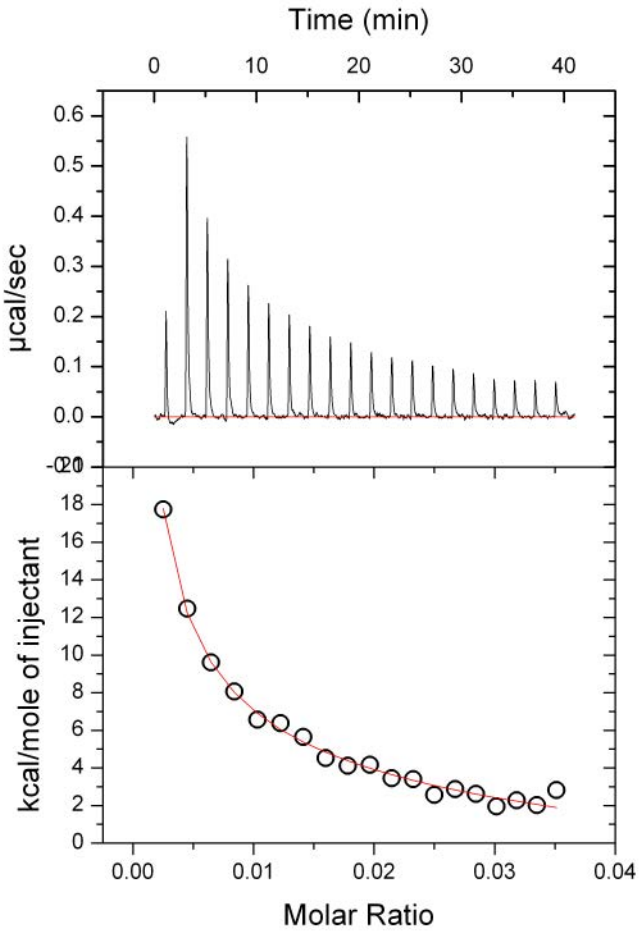
- Titrations in different buffers indicates the net flux of protons.
- Differences in Δv on mutation of protein or between ligands could indicate different binding sites or changes to binding mode.



Overcoming drug resistance in HIV-1 chemotherapy: the binding thermodynamics of Amprenavir and TMC-126 to wild-type and drug-resistant mutants of the HIV-1 protease. Ohtaka et al., Protein Sci. 2002 8:1908-16.

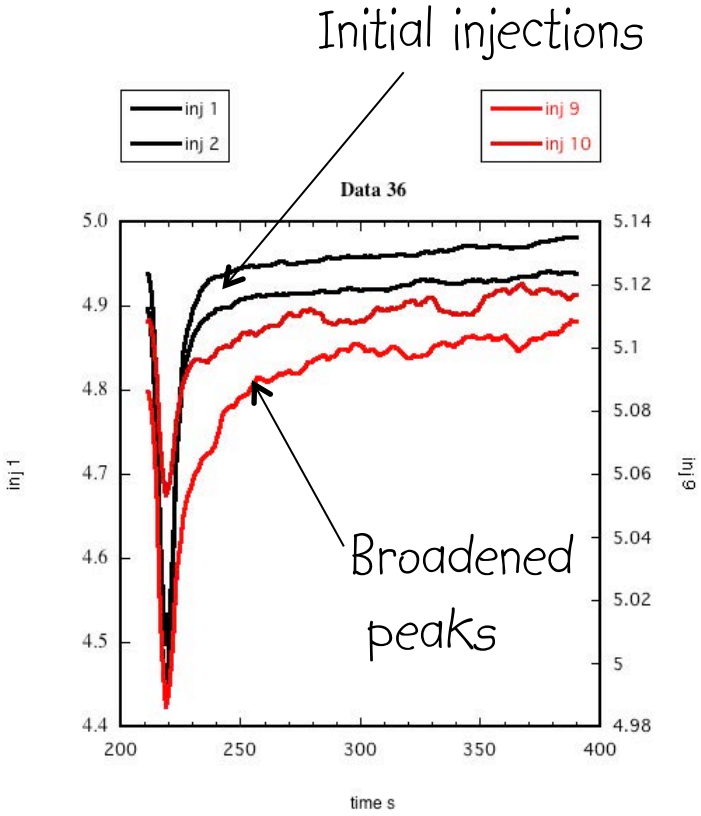
Oligomerisation processes (self association)

- Simply titrate (dilute) protein into buffer (fully recoverable)
- Fit, in this case, to dimer dissociation model
- Suitable for uM kd's
- More complex dissociations possible, hetro-dimer, tetramer etc



Binding kinetics from standard ITC binding experiments?

- ITC measures the rate of heat production (differential power) and so potentially has kinetic information.
- When k_{obs} (k_{on}+ k_{off}) becomes slower than the instrumental response the ITC peaks become broader
- Normally this occurs as the system 'saturates' and k_{on} becomes slow due to limited free sites
- KinITC software*



* https://www.affinimeter.com/site/kinitc-2/

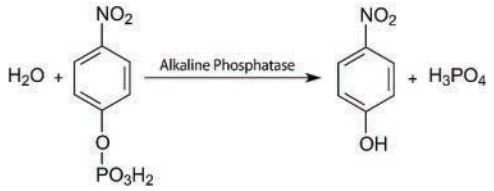
J. Am. Chem. Soc., **2012**, *134* (1), pp 559–565

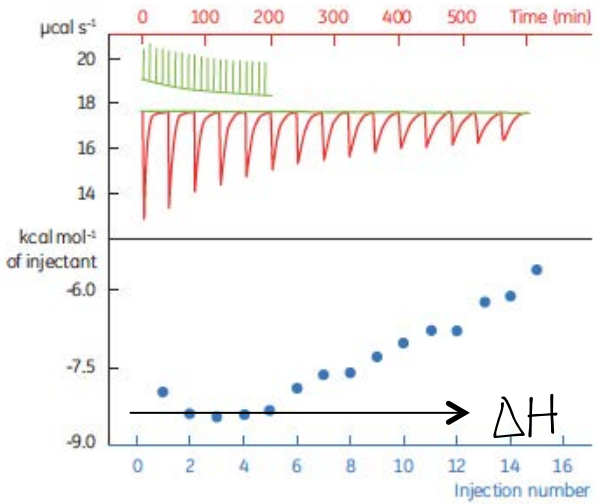
Enzyme / Reaction Kinetics by ITC

A universal* method ?

Non-optical, no assay development or coupled reactions

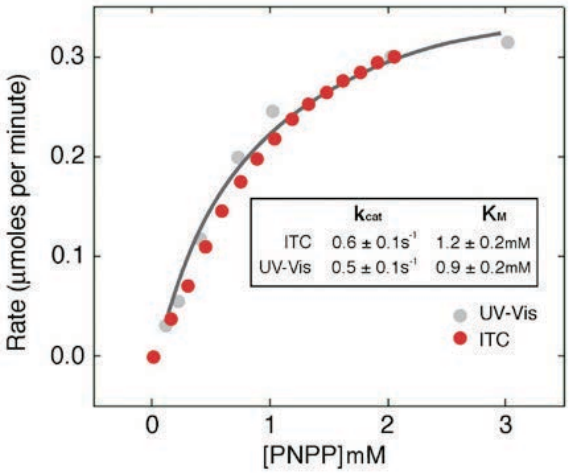
Hydrolysis of pNPP by PPI-γ phosphatase

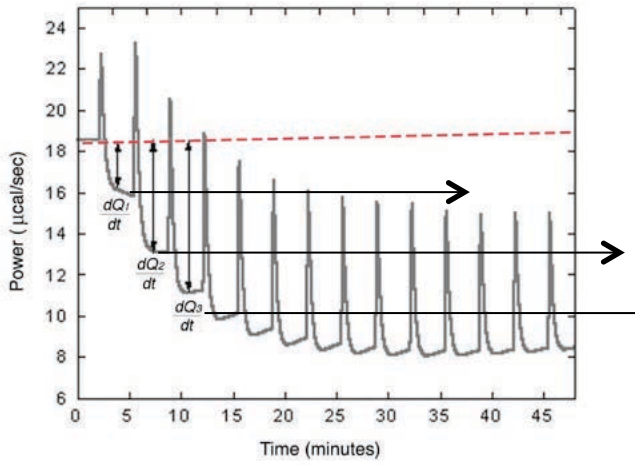




uM enzyme long duration complete turnover

Rate=(1/ΔH)*dQ/dt





nM enzyme short duration initial rate of turnover based on ΔH

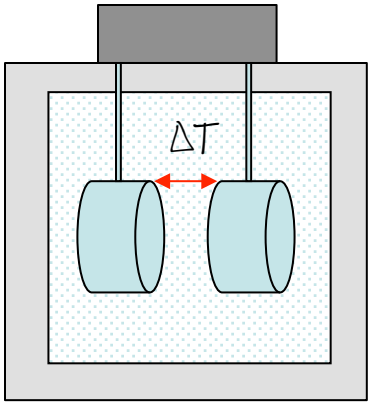
dQ/dt

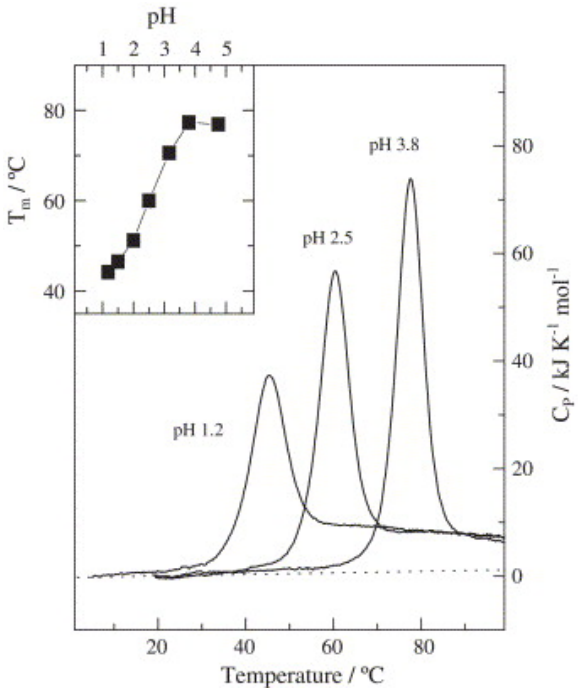
Demarse et al., Methods Mol Biol. 2013;978:21-30.

DSC

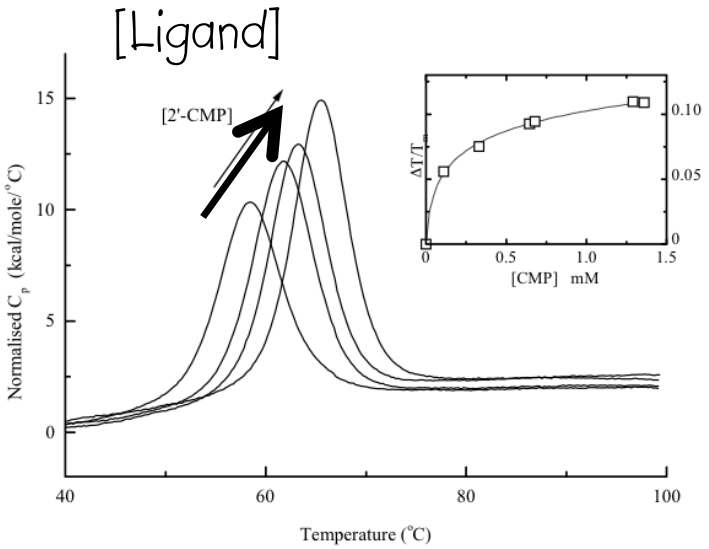
Typical DSC Experiment

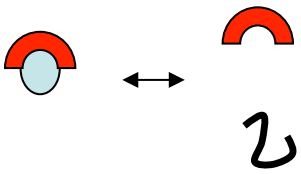
- CAP DSC instrument; robotic loading and operation
- Active cell volume ~130 uL
- 360 ul of a 0.2 – 1 mg ml^{-1} macromolecule
- Temperature range (5-125°C) and scan rate (0.1-2.5 °C/min) variable
- Gives thermal stability (Tm) and enthalpy under different conditions or for mutants



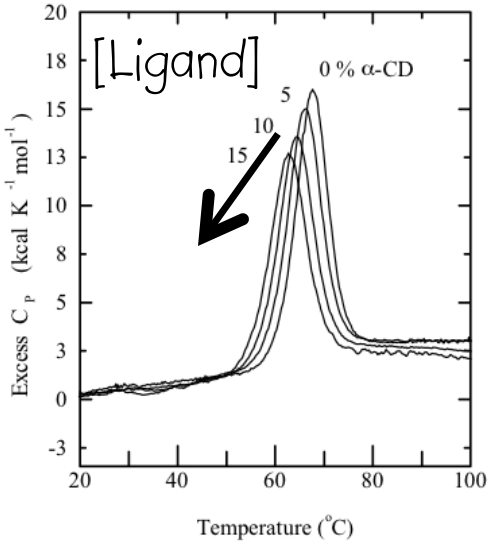


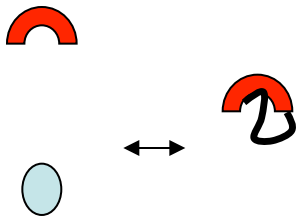
Ligand induced stabalisation or destabalisation





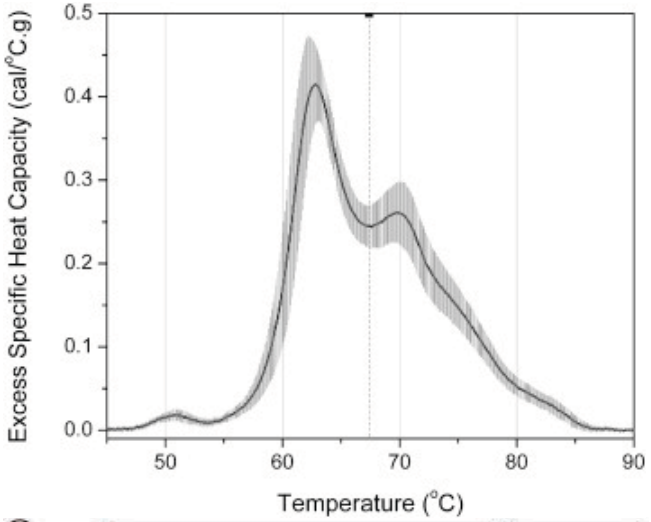
- Mass action increases or decreases the stability as ligands bind to native or denatured state

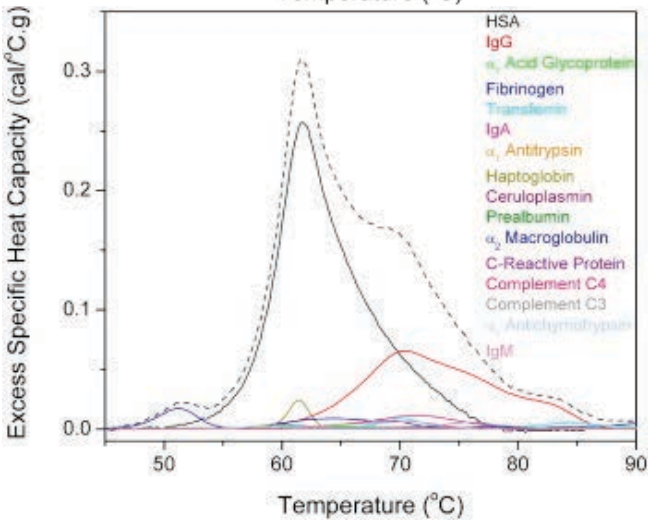




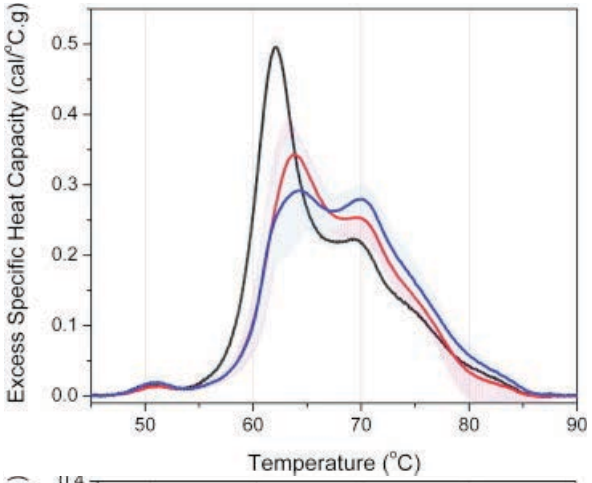
DSC and Complex mixtures

Normal human plasma n=15

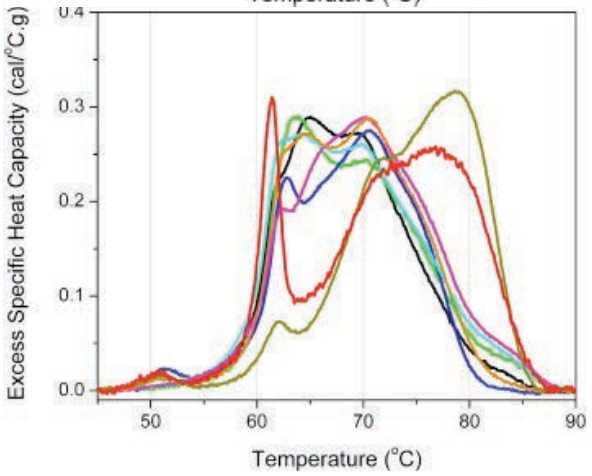




Plasma from patients with a range of cancers and diseases



Blue invasive cervical cancer

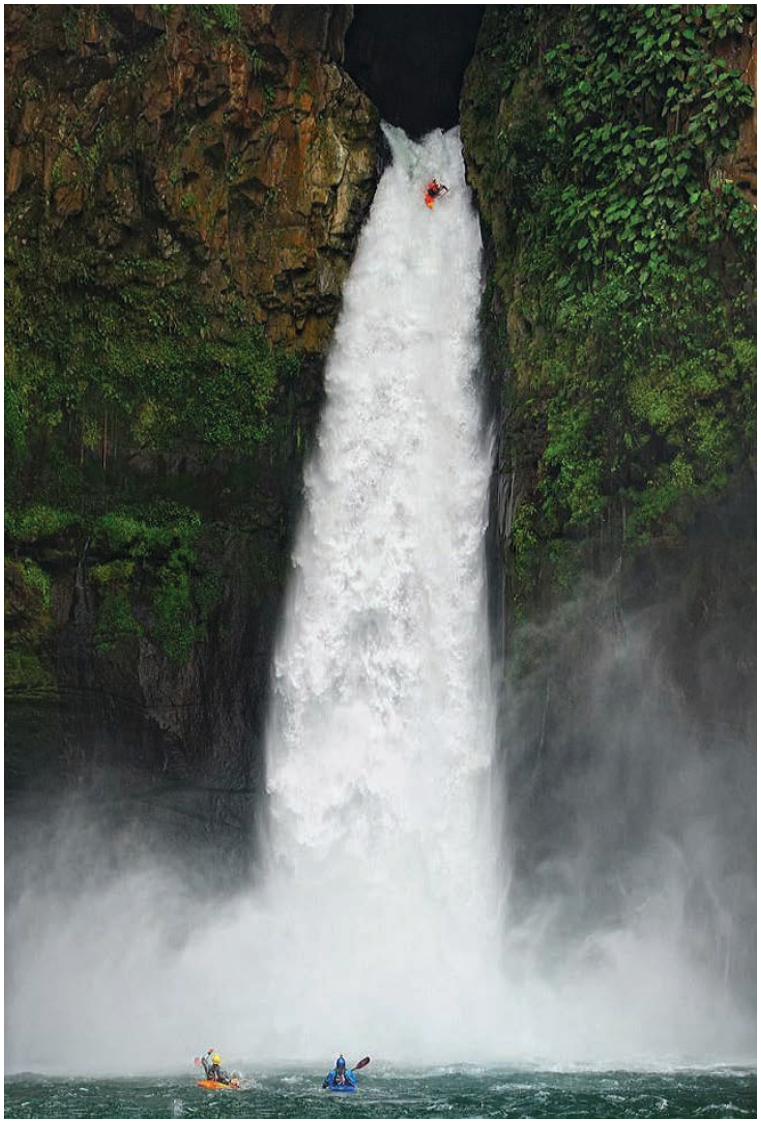


Dark yellow lyme disease

Garbett et al., Experimental and Molecular Pathology Volume 86, Issue 3, June 2009

Summary

- Calorimetry is a label free and very general method for any biomolecular equilibrium .... And not just proteins!
- ITC mainly Binding (Kd, n, ΔH, ΔS, ΔCp, Δprotonation)
- DSC mainly thermal stability (Tm, ΔH, ΔCp, ΔH_{vH} and mechanism, Δprotonation)
- Changes in Tm indicate ligand binding
- Many 'other' uses for ITC and DSC (aggregation, enzyme kinetics, diagnostics, etc.)



Enthalpy and Entropy changes are difficult to interpret in isolation since they include contributions from many processes in the system....

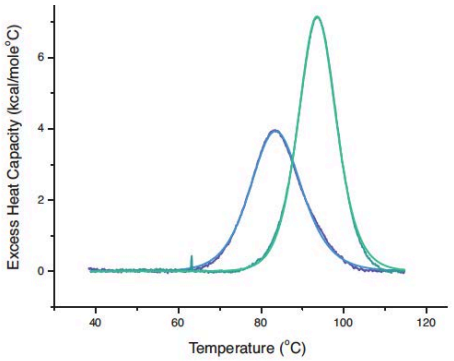
Changes in conformation

Changes in dynamics

Changes in charge

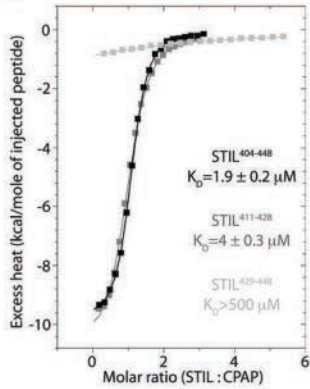
Contributions of water (Proteins may be big but there is a lot of water!)

Some examples of LMB work employing some calorimetry



Ubiquitin Ser65 phosphorylation affects ubiquitin structure, chain assembly and hydrolysis.
Wauer T, Swatek KN, Wagstaff JL, Gladkova C, Pruneda JN, Michel MA, Gersch M, **Johnson CM**, Freund SM, Komander D. EMBO J. 2015 Feb 3;34(3):307-25

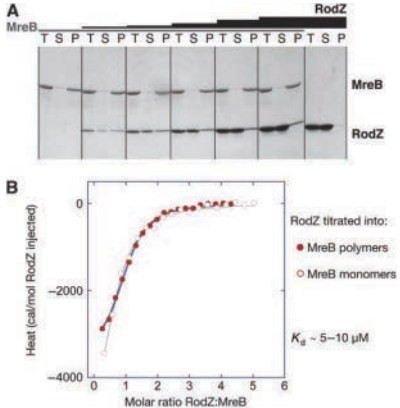
Bacterial actin MreB forms antiparallel double filaments.
van den Ent F, Izoré T, Bharat TA, **Johnson CM**, Löwe J. Elife. 2014 May 2;3:e02634



Crystal structures of the CPAP/STIL complex reveal its role in centriole assembly and human microcephaly.
Cottee MA, Muschalik N, Wong YL, **Johnson CM**, Johnson S, Andreeva A, Oegema K, Lea SM, Raff JW, van **Breugel** M. Elife. 2013 Sep 17;2:e01071

The hepatitis B virus preS1 domain hijacks host trafficking proteins by motif mimicry.
Jürgens MC, Vörös J, Rautureau GJ, Shepherd DA, Pye VE, Muldoon J, **Johnson CM**, Ashcroft AE, Freund SM, Ferguson N. Nat Chem Biol. 2013 Sep;9(9):540-7

Bacterial actin MreB assembles in complex with cell shape protein RodZ.
van den Ent F, **Johnson CM**, Persons L, de Boer P, Löwe J. EMBO J. 2010 Mar 17;29(6): 1081-90



Questions, discussion, training, advice....

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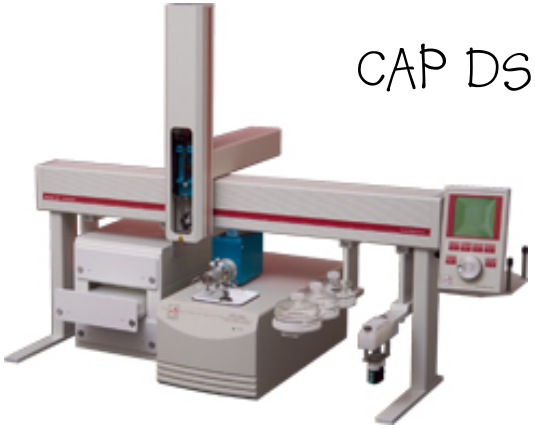
Xtn 7890, 7891

Office 2S005

Interactions lab 2S110

CAP DSC



Manual ITC 200

Auto ITC 200