Introduction to MicroCalorimetry

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Why Microcalorimetry?

Microcalorimetry is the **universal detector** of heat

- Heat is **generated** or **absorbed** in every chemical process

Very easy to do

- Thermal measurements over a wide variety of solution conditions and temperatures

No molecular weight limitations

In-solution

Label-free

Non-optical

Many publications and references

With Isothermal Titration Calorimetry, You Can…

Measure binding affinity between any two biomolecules

Determine binding stoichiometry

Detect multiple binding events

Measure thermodynamics of binding

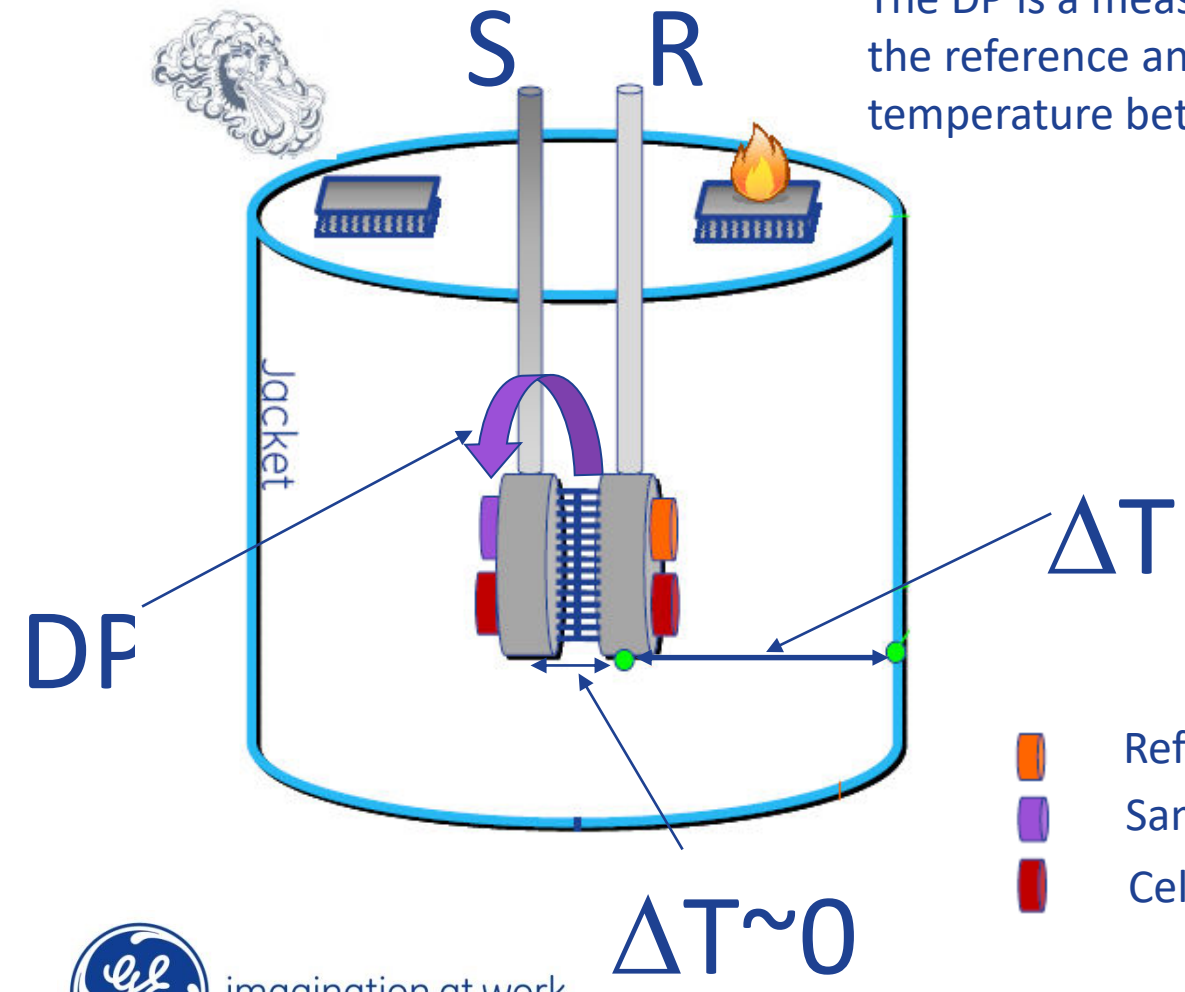
Determine mechanism of action

Validate other assays

Characterize structure-activity relationship

- Drug discovery and development
- Effects of mutagenesis
- Ligand specificity

How Do They Work?



The DP is a measured power differential between the reference and sample cells to maintain a zero temperature between the cells

- Reference Calibration Heater
- Sample Calibration Heater
- Cell Main Heater

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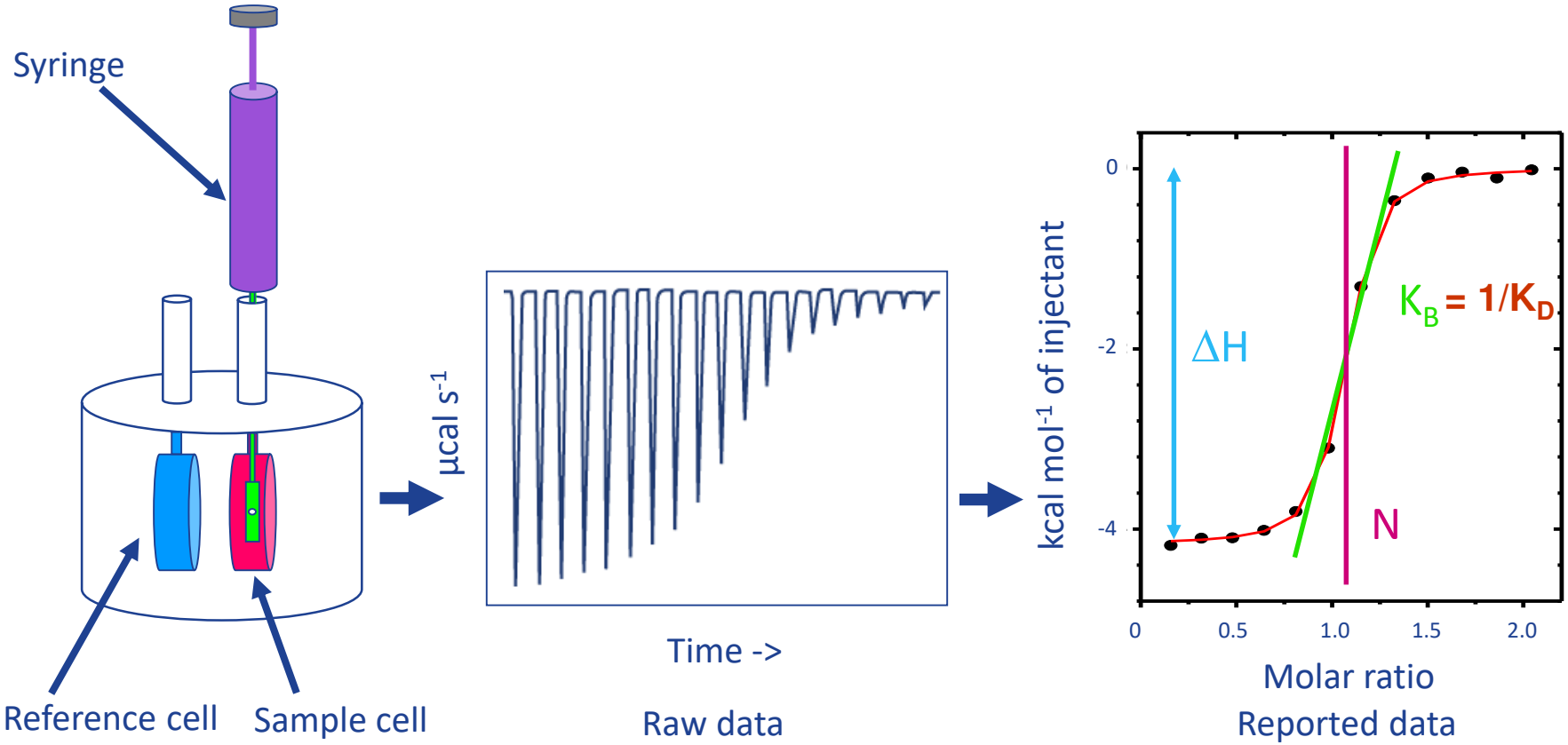
Complete picture of binding in a single experiment!



The Expressions

$$K = \frac{[MX]}{[M] \cdot [X]} \rightarrow [M] = M_t - [MX] \quad \Theta = [MX]/M_t \rightarrow K = \frac{\Theta}{(1-\Theta)[X]}$$

$$X_t = [X] + n\Theta M_t$$

total *X* = free *X* + *X* bound to *M*

Combining equations and elimination of [X] yields the quadratic equation:

$$\Theta^2 - \Theta\left[1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t}\right] + \frac{X_t}{nM_t} = 0$$

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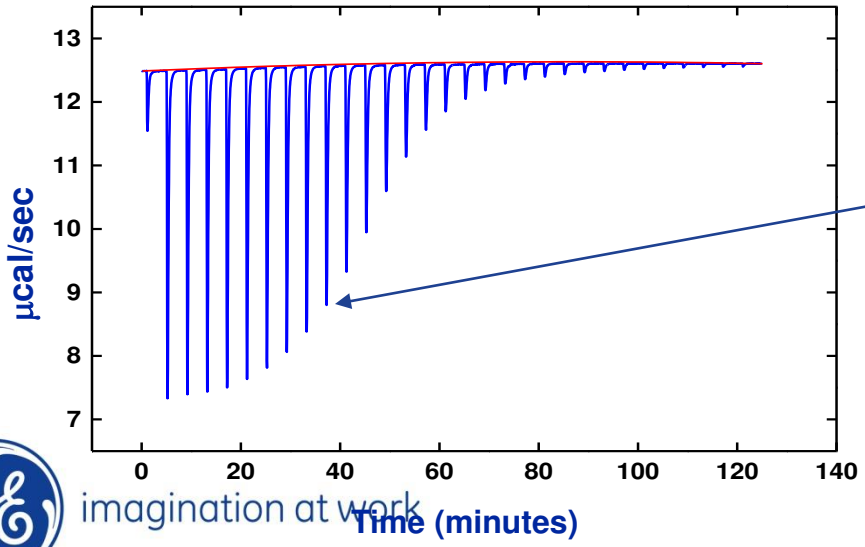
The heat released or consumed due to complex formation is proportional to the amount of compound ($M_t \cdot V_0$), the fraction of complex formed (Θ), the number of sites (n), and the enthalpy of complex formation (ΔH):

$$Q = n\Theta M_t \Delta H V_0$$

Inserting Θ from equation above yields

$$Q = \frac{nM_t \Delta H V_o}{2}\left[1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t} - \sqrt{\left(1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t}\right)^2 - \frac{4X_t}{nM_t}}\right]$$



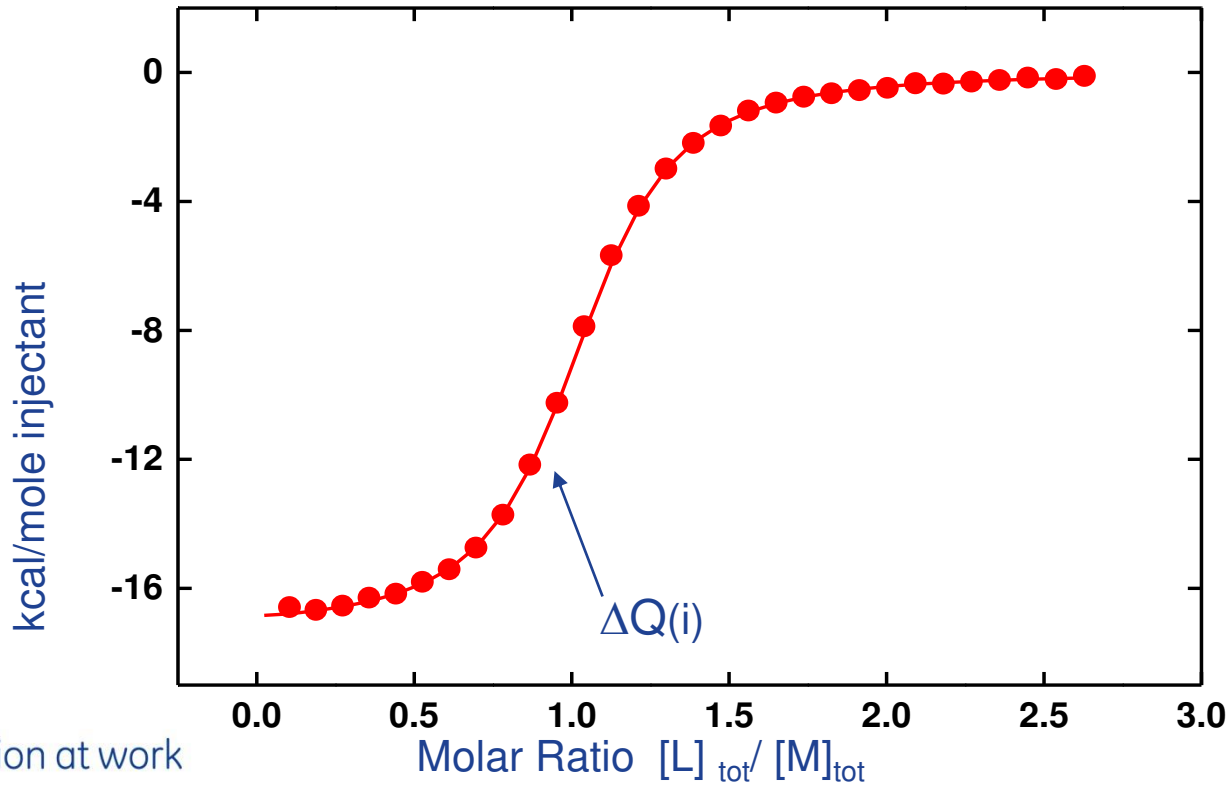
Q(i) = sum of all peak areas up to i^{th} injection

$$Q = \frac{nM_t \Delta HV_o}{2}\left[1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t} - \sqrt{\left(1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t}\right)^2 - \frac{4X_t}{nM_t}}\right]$$

For each individual injection:

$$\Delta Q(i) = Q(i) + \underbrace{\frac{dV_i}{V_o}\left[\frac{Q(i) + Q(i-1)}{2}\right]} - Q(i-1)$$

Small correction factor due to small volume dV_i expelled from cell



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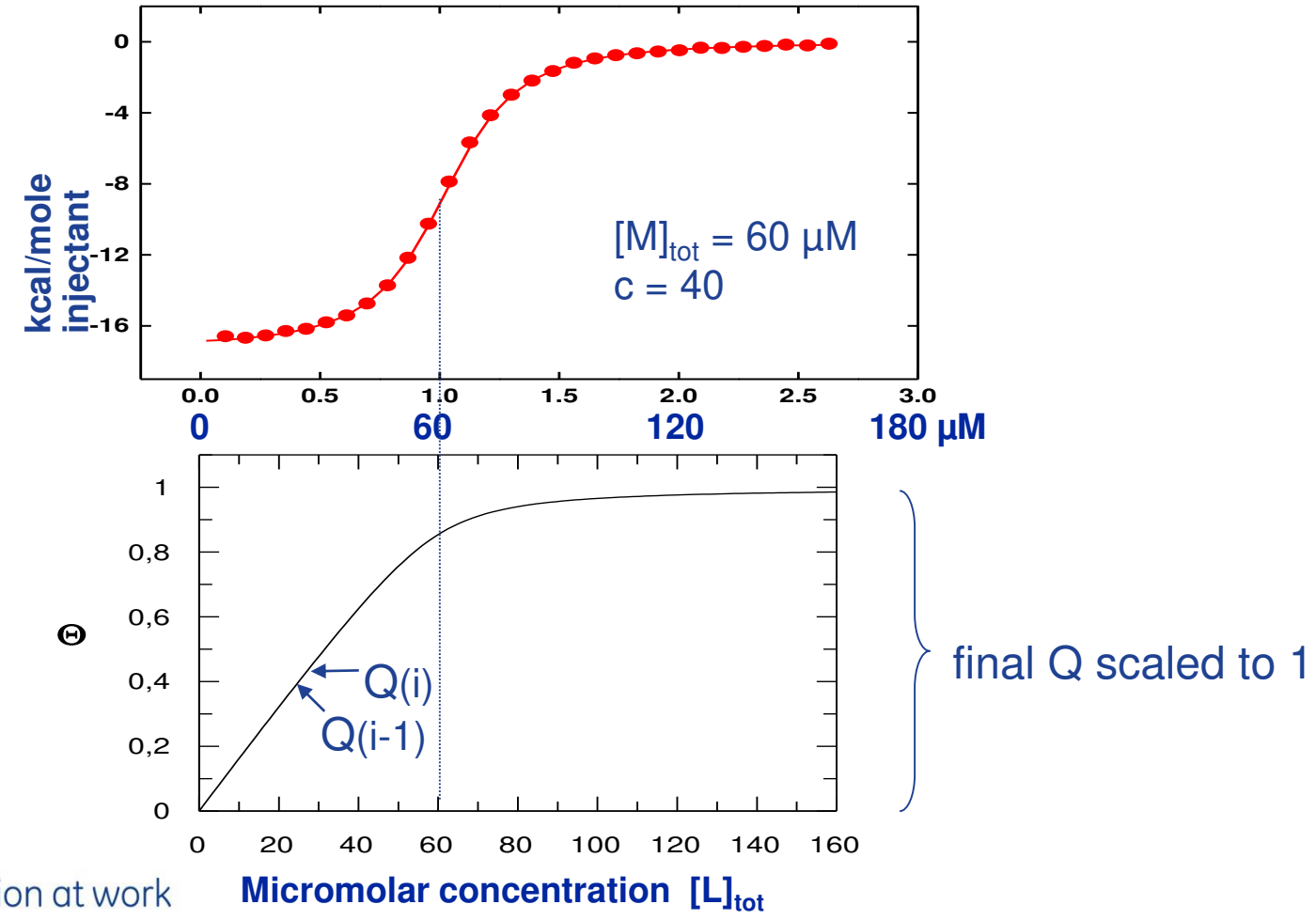
ΔQ_i *versus* Q_i



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Thermodynamics

$\Delta G = \Delta H - T\,\Delta S$ $\Delta G = -RT\,\ln K_B$

K_B (or K_A) – binding constant – relative strength of interaction

K_D - equalibrium dissociation constant = $1/K_B$

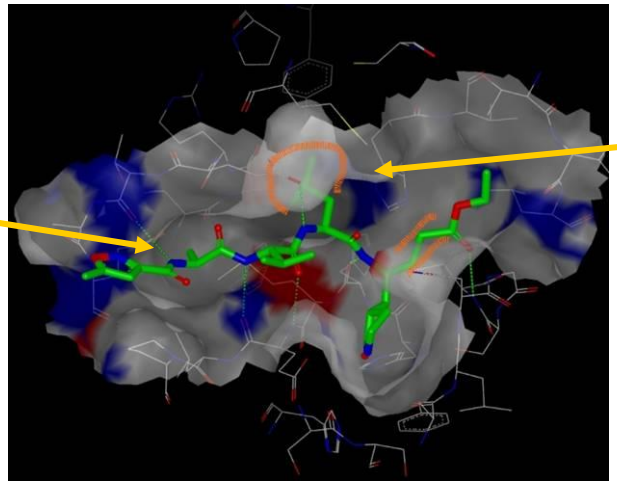
Microcalorimetry provides a total picture of binding energetics

Overall binding affinity K_D correlates with IC_{50} or EC_{50}.
This is directly related to ΔG, the total free binding energy

ΔH, enthalpy is indication of changes in hydrogen and van der Waals bonding

-TΔS, entropy is indication of changes in hydrophobic interaction and conformational changes

N, stoichiometry indicates the ratio of ligand-to-macromolecule binding



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

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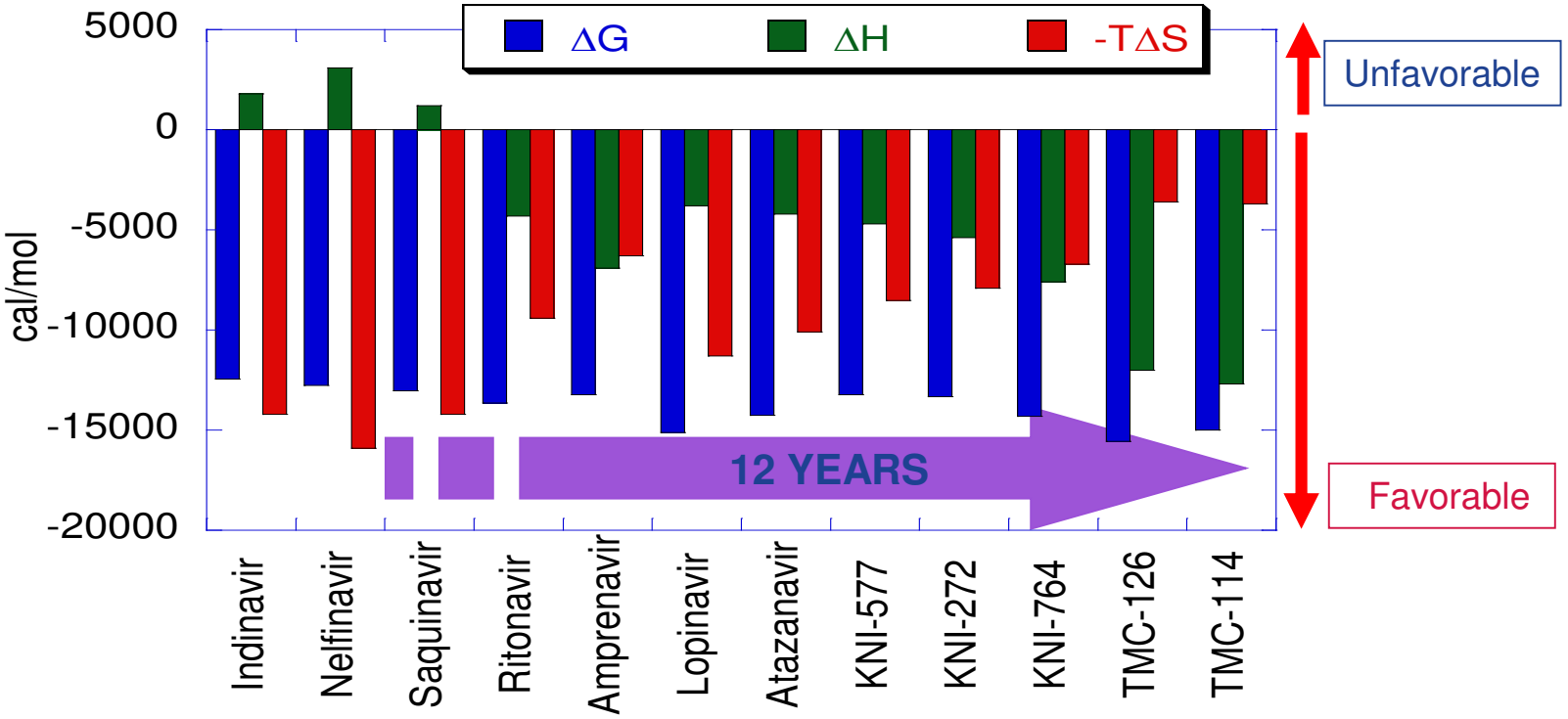
Evolution of HIV-1 protease inhibitors over 12 years



Improvement in hydrogen and van der Waals binding (ΔH) from +2 to -12.5 kcal/mole

Ohtaka and Freire, Prog Biophys Mol Biol 88, 193-208 (2005)

Practicalities

The Major Steps To Good Quality Data

Start with clean instrument

1/ Good experimental Design-use correct concentrations

2/ Good sample preparation

3/ Accurate concentration determination

4/ Use correct run parameters

5/ Perform appropriate controls experiments

6/ Data Analysis and model choice-another tutorial

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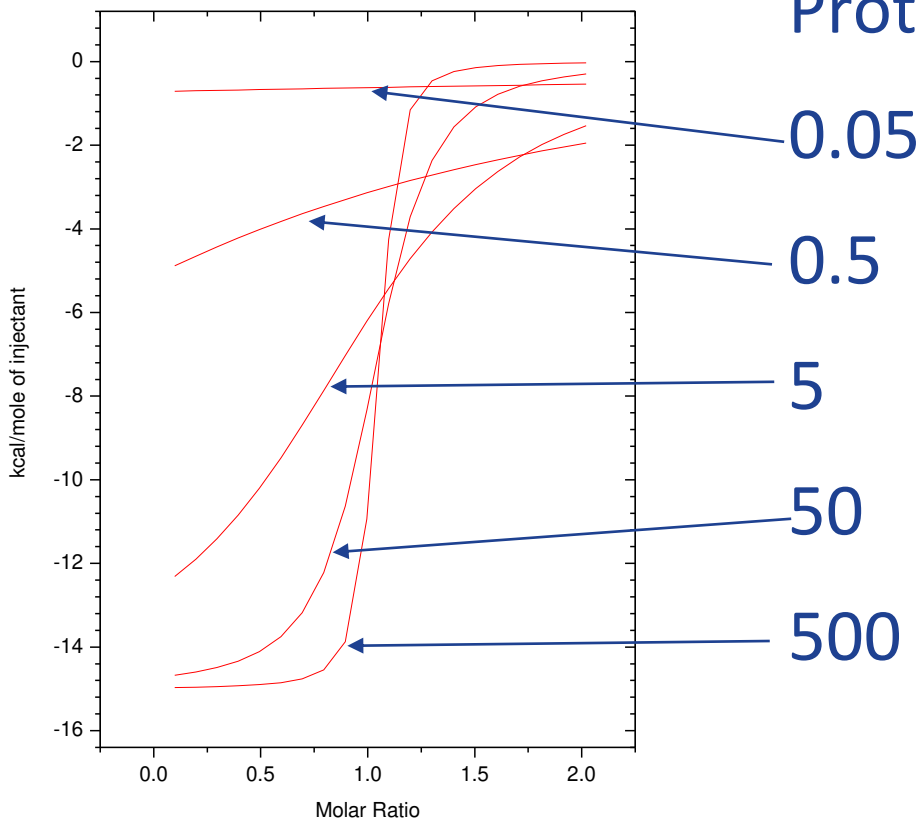
C Values



Protein in cell/KD*N=C=

0.05

0.5

5

50

500

C = 10-100 very good

C = 5-500 good

C = 1-5 and 500-1000 OK

C = < 1 and > 1000 bad

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Good Experimental Design Table

K_D (Biacore) µM	[Protein] µM	[Compound] µM	[Protein] / K_D
<0.5	10	100	>20
0.5-2	30	300	15-60
2-10	50	500	5-25
10-100	30	40*K_D (Biacore)	0.3-3
>100	30	20*K_D (Biacore)	<0.3

} Fixed stoichiometry

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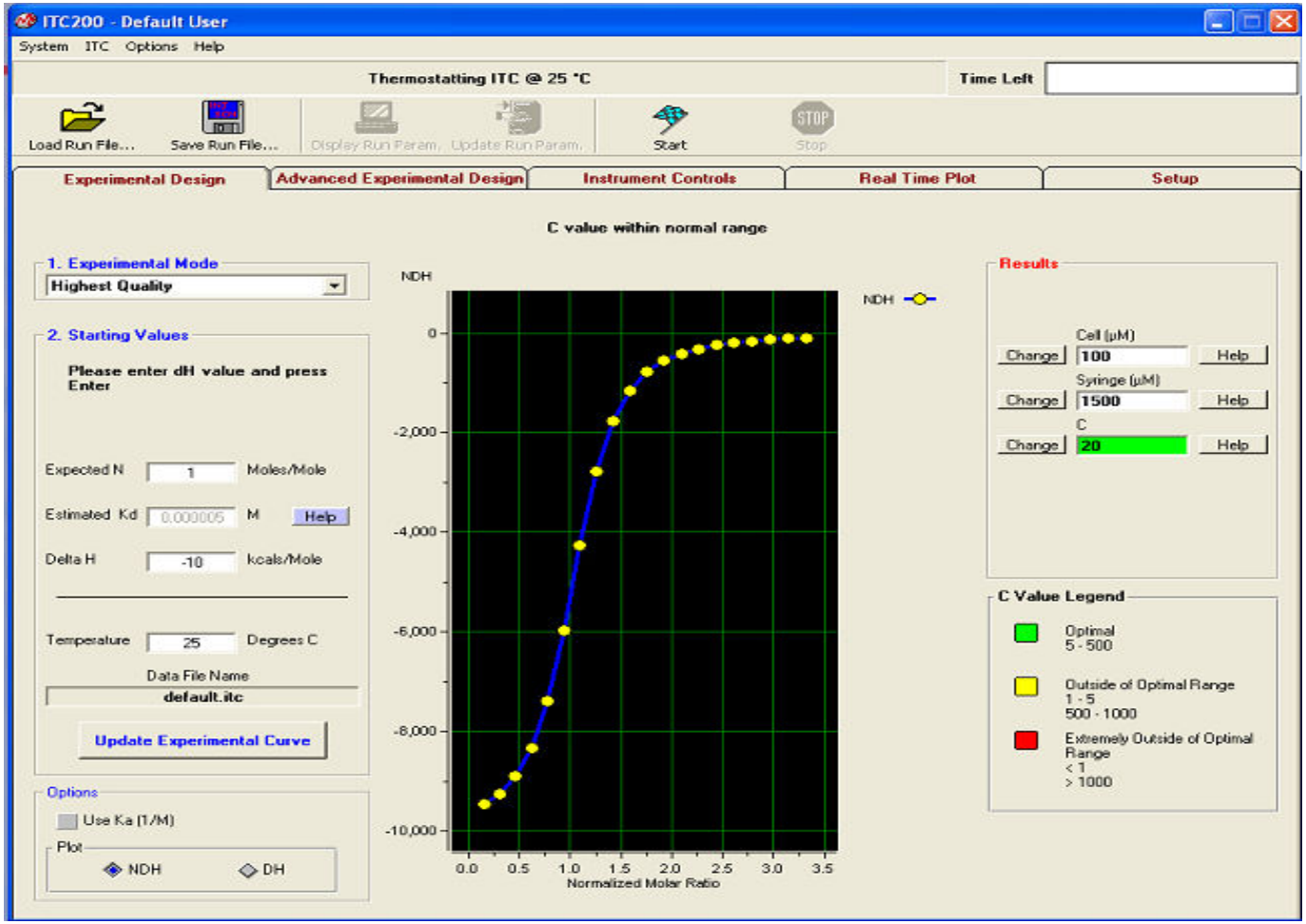
Binding Curve Simulation



OR.. With little prior knowledge

Good Starting Conditions

100μM Ligand in the Syringe and

10μM molecule in the cell

12 x 3μl injections

Detect K_ds of 10 μM to 10 nM

Ideal for K_ds of 2 μM 100 nM

Minimum heat requirement ~ 5 μM (10 μM for iTC_{200})

Run Parameters-

- Experimental Parameters-Default
  - Temp-25 C
  - Number of injections -12 –18
  - Reference power-5 μcal/sec
  - Initial delay-60 sec
  - Stir speed-300 rpm VP-ITC , 1000 rpm (1500 for SIM) iTC_{200}
  - Feedback Mode-High-
    - No feedback will give better S/N but will take a little longer (see time between injections) –normally use when working with small heats

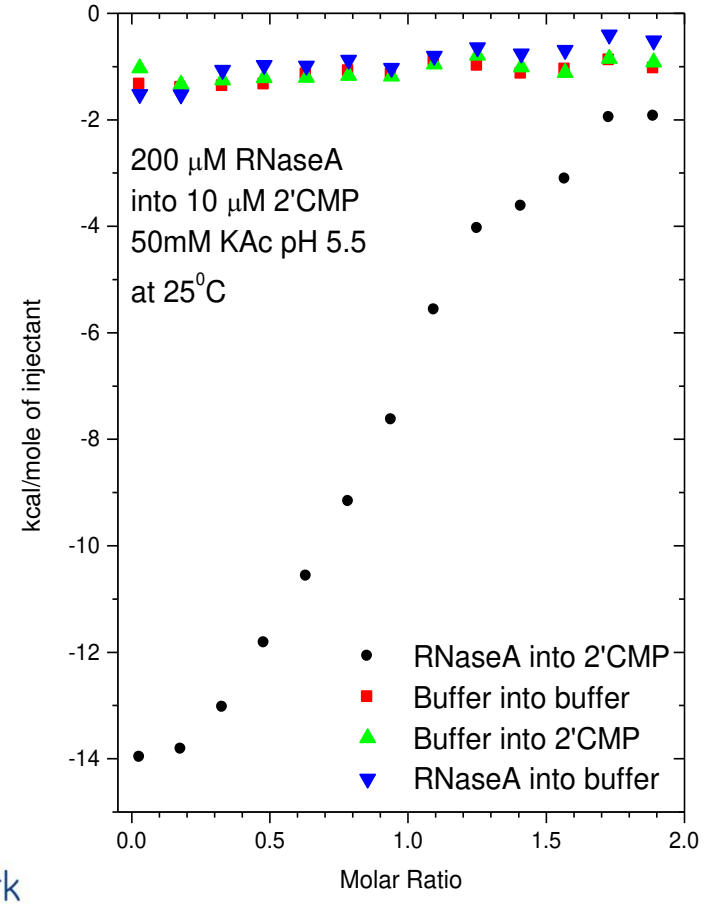
Run Parameters-iTC200

- Injection Parameters
  - Volume-Typical 2-3 μl (range 0.1-38 μl)
  - Duration-2*vol (μl) e.g. 3 μls injected over 6 secs-default
  - Spacing-Typical 120 secs-may want to extend to 180 secons or more if using no feedback with large heats-default
  - Filter period –5 secs-the time span of data acquisition for data averaging-default

Choice of buffer

- Avoid DTT
  - ✓ Unstable and undergoes oxidation
  - ✓ High background heat
- Use β-mercaptoethanol & TCEP
- TCEP is not stable in phosphate buffer
- Use conditions in which your protein is ‘happy’

Control Experiments



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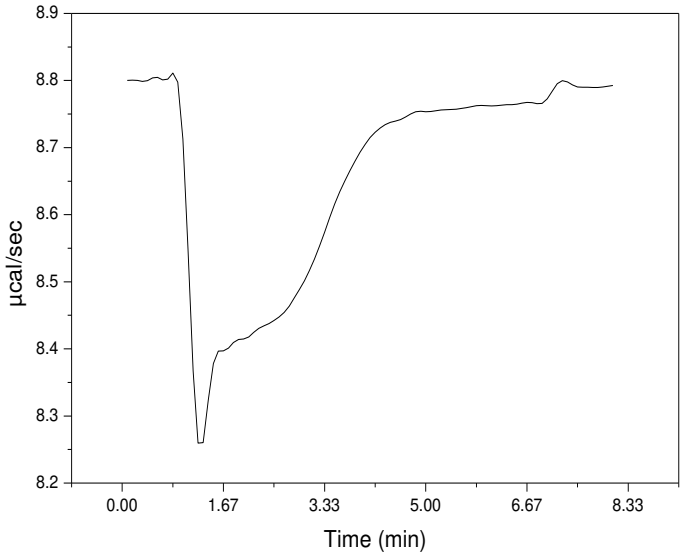
Data Analysis Models Available

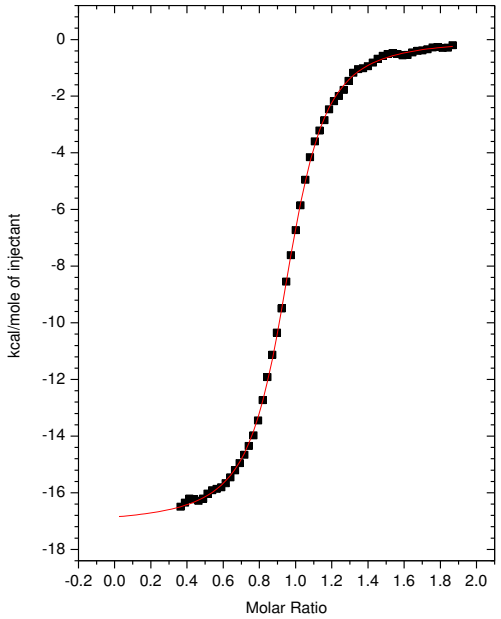
- One set of sites
- Two set of sites
- Sequential binding
- Competitive binding
- Dimer dissociation
- Enzyme Kinetics

Single Injection Method iTC_{200}

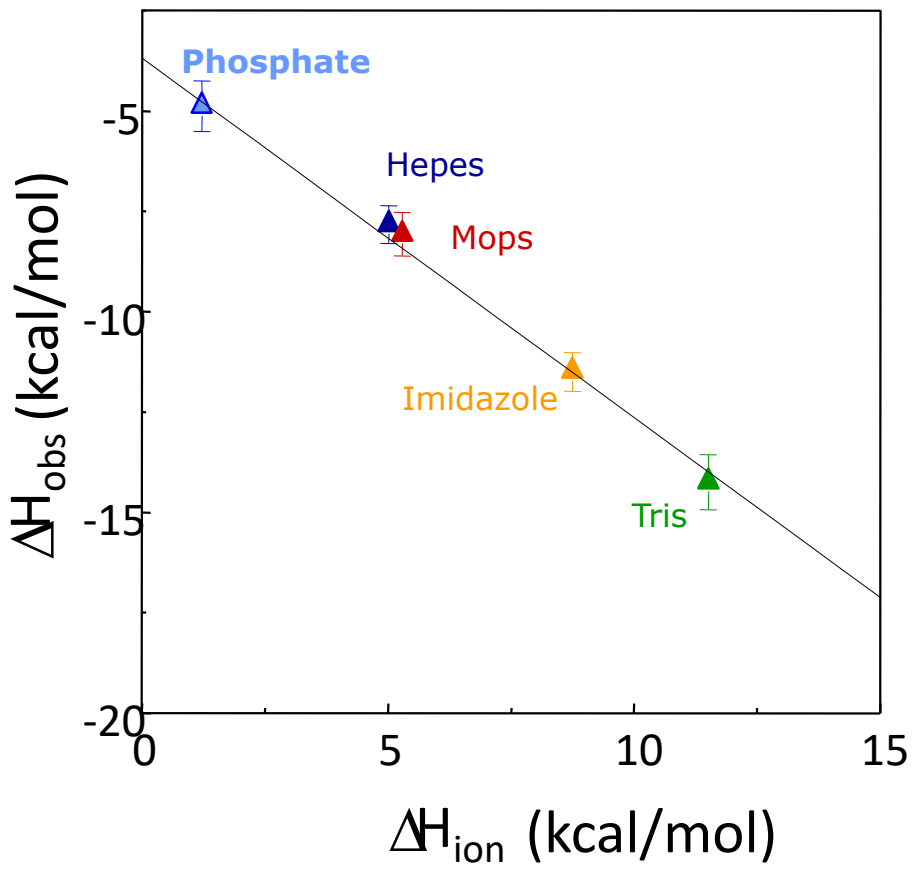
High Speed Mode



High Quality Data in 7 minutes



ΔH_{obs} versus buffer heat of ionization



All reactions at same pH
Slope: # protons released (negative value)
Y intercept: ΔH_{int} of binding, buffer-independent

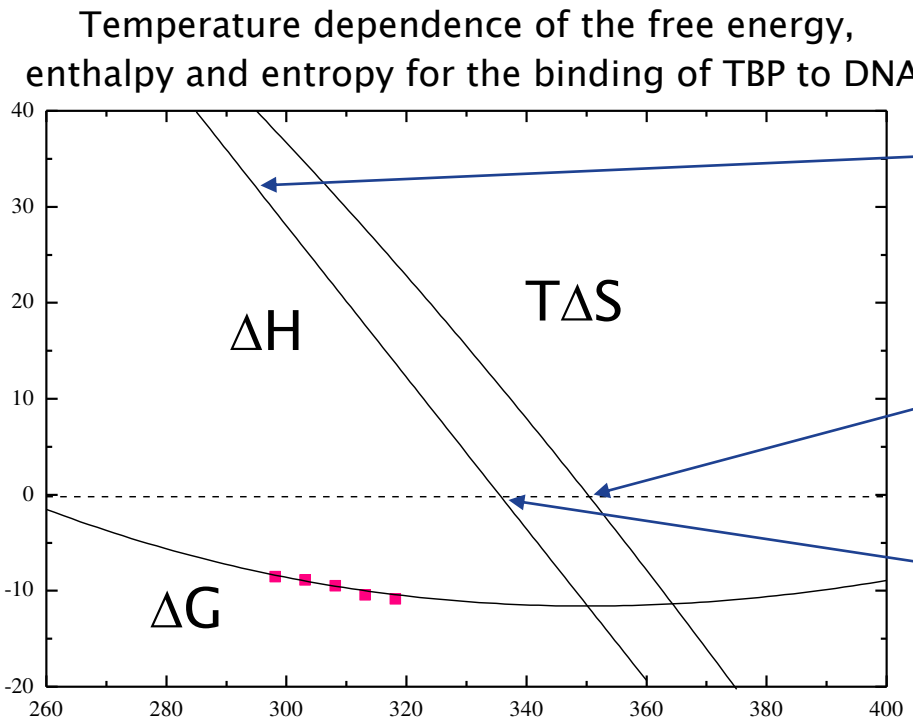
Different pH can have different plot

If slope = 0, then no buffer effect at that pH

$$\Delta H_{obs} = \Delta H_{int} + n\,\Delta H_{ion}$$

Evaluation of Linked Protonation Effects in Protein Binding Reactions Using Isothermal Titration Calorimetry, Biophysical J., 1996, Brian Baker et al.

The Energetics



$$\Delta G(T_0)=\Delta H(T_0)-T_0[[\Delta H(T)-\Delta G(T)]/T+\Delta C_P \ln(T_0/T)]$$

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Experimental conditions

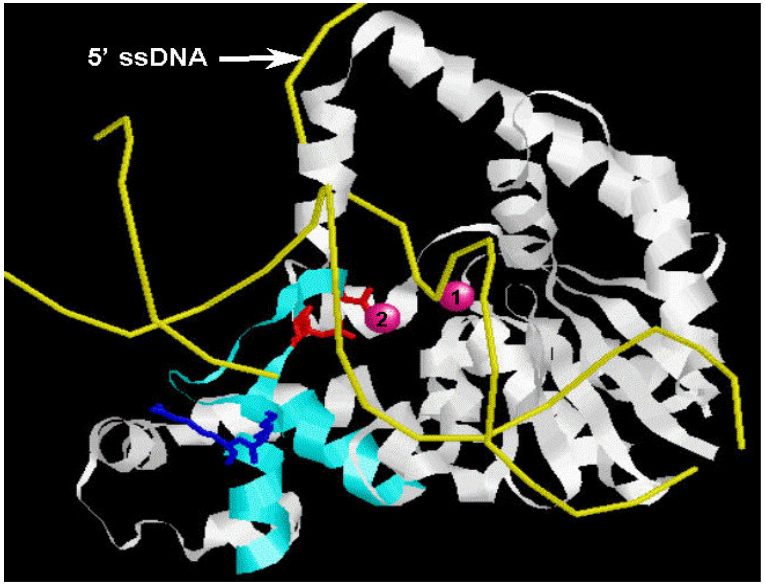
For full characterization of binding interaction, need to do experiment at different conditions

- Temperature
- pH
- Buffer
- Ionic strength

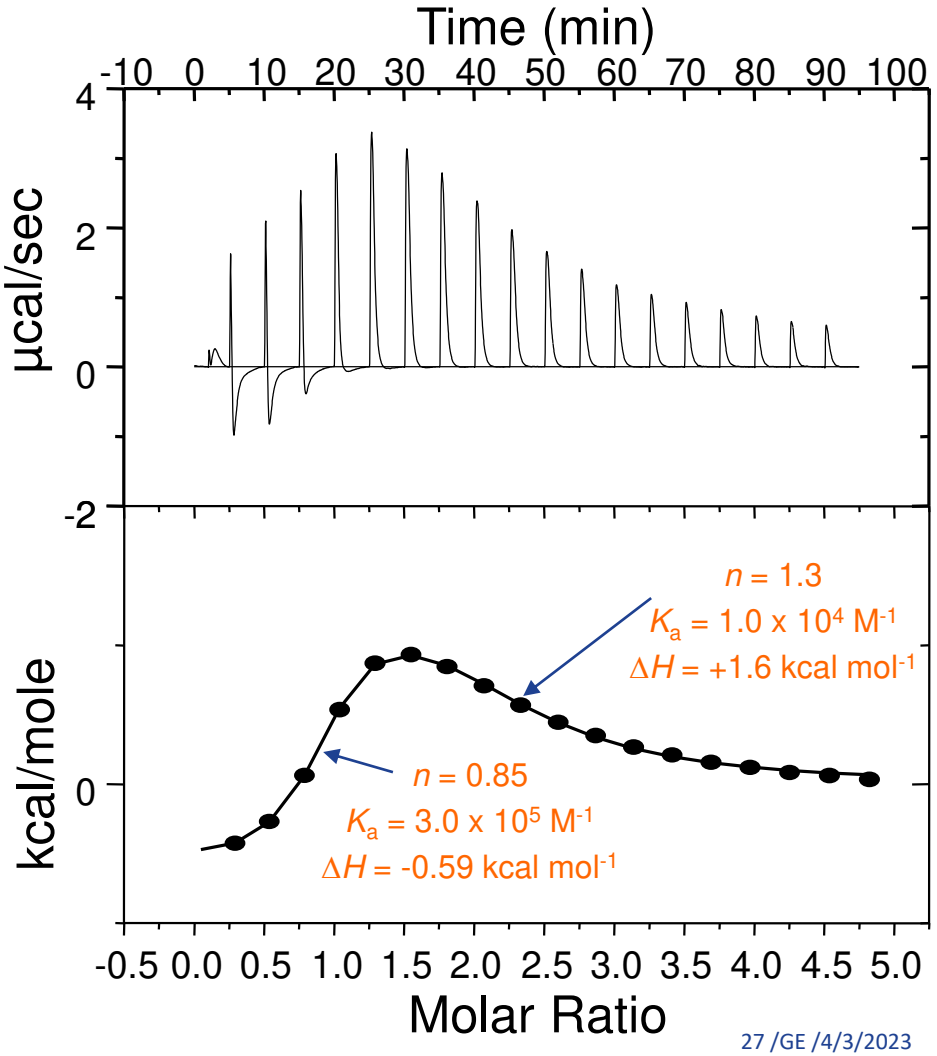
For comparison studies (e.g. mutant protein studies, drug binding screening) need to do experiments at identical conditions

Multiple Binding Sites

ITC shows differential binding of Mn(II) ions to WT T5 5’ nuclease

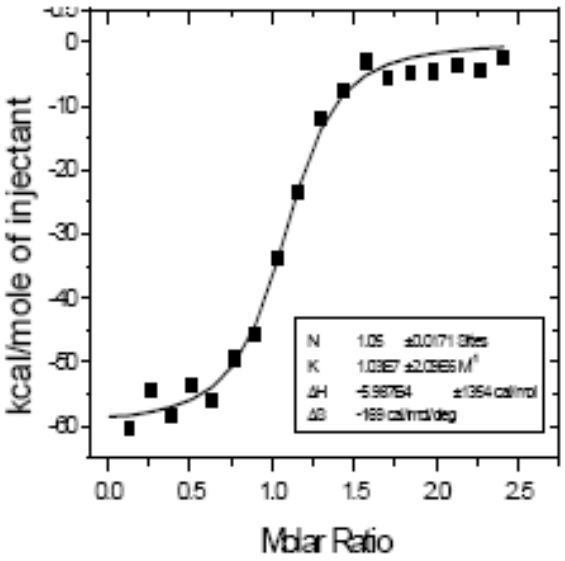


Feng, et al, Nat. Struct. Mol. Biol. 11, 450-456 (2004)



Assessment of protein quality by MicroCal™ iTC_{200}

Peptide binding to Batch 1 protein

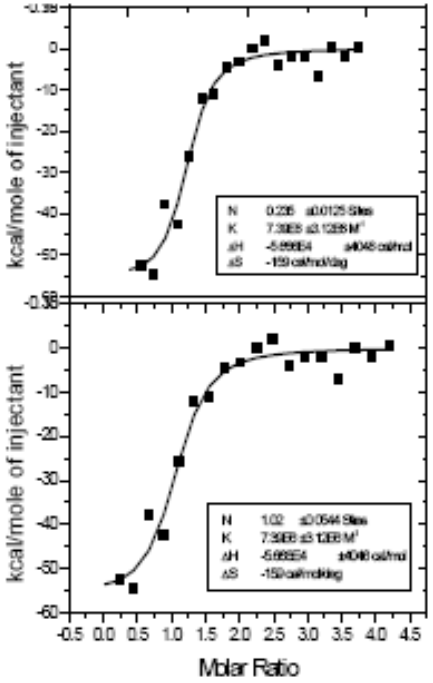


N = 1.05

K_D = 97 nM

100% of Batch 1 protein Active based on Stoichiometry

Peptide binding to Batch 2 protein



50 µM Peptide
10 µM Protein X

N = 0.235
K_D = 135 nM

Re-analyzed
2.3 µM Protein X

N = 1.02
K_D = 135 nM

23% of Batch 2 protein Active based on Stoichiometry

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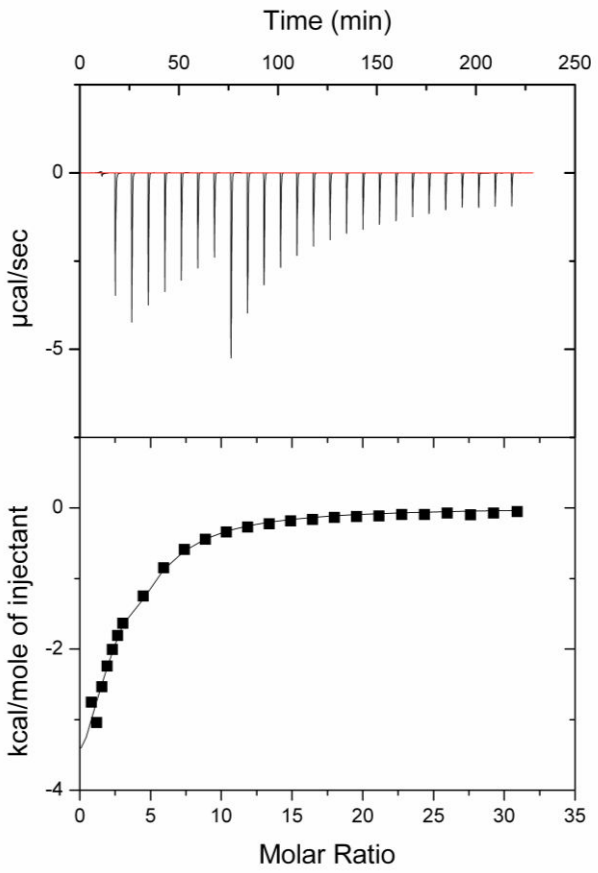
GE Healthcare Application Note (2010).
Data courtesy of L. Gao, Hoffmann-LaRoche

Determine excipient mechanisms: protein-excipient binding with iTC_{200}



Binding of polysorbate-80 to Protein X

K_B = 1430 ± 260 M^{-1} (0.7 mM)

N = 2.6 ± 0.3

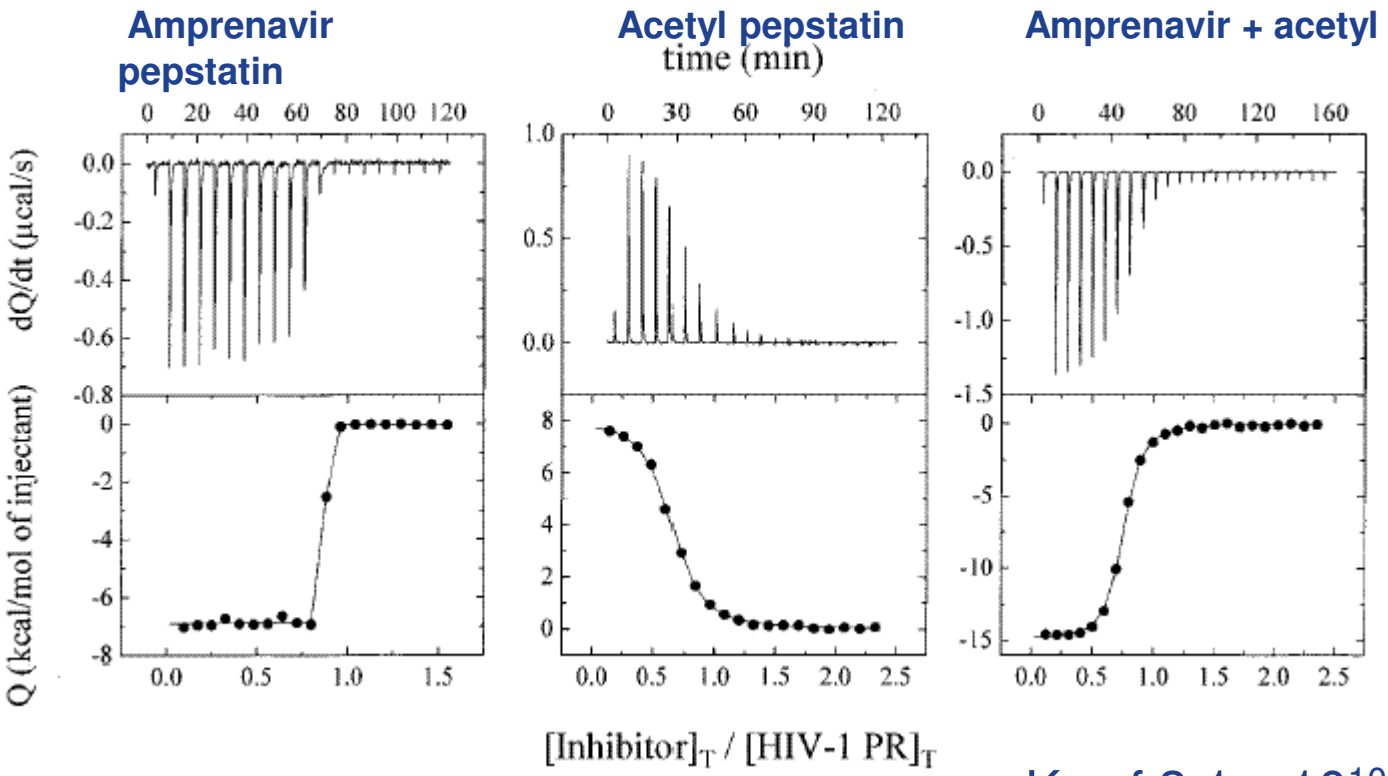
Binding saturation of ~10 moles of polysorbate-80 per mole of Protein X

Weak interaction: polysorbate-80/Protein X complex more likely to dissociate in vivo

ITC data suggests minimum excipient concentration needed to stabilize Protein X in formulation

Data from Woods et al, MicroCal application note 2010, GlaxoSmithKline

Displacement/Competitive ITC – HIV-1 Protease - Inhibitor Binding



Unable to determine K_B

K_B of 3.1 x 10^{10} M^{-1}

Ohtaka, et al, Protein Sci. 11, 1908-1916 (2002)

ITC Provides Insights on:

- K_D
- Binding stoichiometry
- Hydrogen bonding alignment
- Conformational changes due to binding
- Hydrophobic interaction information
- Solvent effects
- Enzyme kinetics
- Minimal assay development, label-free

Free energy change

- ΔG is change in free energy
- $\Delta G \leq 0$ for spontaneous process
- More negative ΔG, higher affinity

Enthalpy change

- ΔH – Negative value for favorable enthalpy change
- ΔH – directly related to number and strength of hydrogen bonds broken or formed during interaction
- Related to conformational changes
- Solvents play a role

Entropy change

- ΔS – positive for entropically driven reactions

Hydrophobic interactions

Solvation entropy (favorable) due to release of water

Conformational degrees of freedom (unfavorable)

Heat capacity

ΔCp – determined by performing ITC experiments at different temperatures

Plot temperature vs. ΔH

Slope is ΔCp