Equilibrium Thermodynamics and Biological Calorimetry

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Most of biology is based around reversible equilibrium processes that are mediated by many individually ‘weak’ non covalent interactions
Biological Reversible Equilibria
For example, protein folding or protein-DNA binding

- At equilibrium the ‘reaction’ position can be represented by the equilibrium constant, $K_{eq}$, which is the ratio of the concentrations (products / reactants) or the ratio of the forward and reverse rate constants

$$K_{eq} = [D]/[N] = k_u/k_f$$

$$K_{eq} = K_a = 1/K_d = [NL]/[N]*[L] = k_{on}/k_{off}$$

Biological Equilibrium Thermodynamics

- The Gibbs Free Energy ($\Delta G$) is a logarithmic representation of the equilibrium position.

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

- It has ‘components’ of ‘Enthalpy’ ($\Delta H$) and ‘Entropy’ ($\Delta S$)

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

- When $\Delta G$ is $-ve$, $\ln K_{eq}$ is $+ve$, so $K_{eq} > 1$ and so the products are in excess (e.g., for $K_a$ this is NL)

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

Josiah Willard Gibbs
1839 – 1903
What are Enthalpy and Entropy?
A Simplified View

- Systems naturally progress to a lower internal energy level; lower enthalpy.
- The change, $\Delta H$, is $-\text{ve}$ 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; $\Delta S$, is $+\text{ve}$ (more ways) equilibrium product favoured $(-T\Delta S$ is $-\text{ve}$)

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

Where does the equilibrium end up?
- Simply the balance of enthalpy and temperature-entropy gives $\Delta G$, and thus defines the equilibrium position.
- Temperature is a factor
- A $-\text{ve} \Delta G$ is ‘favourable’ and can be obtained even when one component is ‘unfavourable’ if the other is ‘favourable’ and larger
Are $\Delta H$ and $\Delta S$ Fixed values?

Changes in heat capacity

- In biological equilibria there are typically large changes in solvation which affects the system heat capacity ($C_p$), 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, $\Delta C_p$
- The integral of $C_p$ or $C_p/T$ from zero K to temperature $T$ represents the enthalpy and entropy levels for a system
- Because of the significant $\Delta C_p$ for biological equilibria the corresponding $\Delta H$, $\Delta S$ (and thus $\Delta G$) are all temperature dependent in a non linear manner

\[
\Delta G = \left[ \Delta H + \Delta C_p (T_2 - T_1) \right] - T_2 \left[ \Delta S + \Delta C_p \ln \left( \frac{T_2}{T_1} \right) \right]
\]

\[
\Delta H = \int_0^\gamma \Delta C_p \, dT
\]

Other than temperature, can anything else affect $\Delta G$?

... 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”
For example....
Protein-protein Interaction with a change in protonation

- The pKa’s of groups change on complex formation
- Equilibrium position changes the proton concentration (although pH change is prevented by the buffer)
- Therefore 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, equilibrium (stability, melting temperature) depends on concentration of the free ligand (the basis of thermal shift high throughput screening)
How do we measure Enthalpy?

Under constant pressure the heat transferred during a process is equal to the enthalpy (ΔH).

Calorimetry (Calor; heat Metrum; measure) is thus a technique that measures directly values of ΔH

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 TΔS (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.
- Applicable to many systems from the molecular to cellular level (so long as material can be put in the cell!)
- 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.
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’

‘Biocalorimetry’

- 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.

* Kd 50nM @ 298K ΔS =0
Power Compensation Biocalorimeters

- Difference in temperature relative to an “identical” reference cell, measured by very precise thermopile, 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)

Calorimetric investigation of ribonuclease thermal denaturation.  
Rapid measurement of binding constants and heats of binding using a new titration calorimeter.
Other (non-calorimetric) ways of measuring Enthalpy

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

- Rearranging the equation (dividing both sides by \(-RT\)) allows enthalpy to be determined from the van’t Hoff plot if we measure \(K_{eq}\) as a function of temperature.

- This is an *indirect* measure so sometimes distinguished from directly measured calorimetric enthalpies by \(\Delta H_{vH}\).

\[ \text{Gradient} = \frac{\Delta H_{vH} \cdot \Delta S}{-RT} \]

* Indirect because the per mol term comes from \(R\) and we apply a two state model

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

- 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 X-ray or NMR), but also changes in solvation, shifts in pKa’s (i.e., changes in protonation), etc.

- This emphasises the totality of molecular forces contributing to the interaction process in solution (which when not resolved in structures are often forgotten or ignored).

- However, components of the total can in some cases be deconvolved giving a simple direct method for their measurement.
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!

\[
\Delta H_{\text{obs}} = \Delta H_{\text{AB}} + \Delta H_{\text{solvation}} \\
\Delta S_{\text{obs}} = \Delta S_{\text{AB}} + \Delta S_{\text{solvation}}
\]

Interactions in Buffers

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

\[
\Delta H_{\text{obs}} = \Delta H_{\text{AB}} + \Delta H_{\text{solvation}} + \Delta H_{\text{ionisation}}
\]
\[
\Delta S_{\text{obs}} = \Delta S_{\text{AB}} + \Delta S_{\text{solvation}} + \Delta S_{\text{ionisation}}
\]
Measuring Protonation Changes (Δv)

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

![Graph showing slope = Δv]


Interpretation of thermodynamics may be (currently) impossible, but there may be qualitative trends....

![Graph showing Log Ka vs. DNA Binding mode]

Allophenylnorstatine inhibitors of plasmepsin II Ruben et al. Chemical Biology & Drug Design 2006, 67, 2-4
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 200 instrument (active cell volume 200 uL)
• 350 uL of ‘target’ 10 - 50 uM* loading cell (275 uL consumed)
• 70 uL of ‘ligand’ 50 – 500 uM* loading syringe (55 uL consumed)
• 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.

* Typical starting concentrations. Actual concentrations depend on $\Delta H$ (signal amplitude), $K_d$ of binding and the type of experiment.
Sources of signal and controls for ITC experiments


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 \( \Theta \) is \( \sim 0 \) then 4 should be corrected with 3 to give 2.

Example: ITC measurement and separate control

600 \( \mu \)M peptide into 45 \( \mu \)M protein or into buffer

Integrate heat from each injection. Subtract control and fit.
Single experiment measurement

500 μM ligand into 30 μM 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 8.8 kcal/mol
ΔS 6.96 cal/mol/deg

*Software provided with Microcal calorimeters
More Data Fitting

- NLLS Fitting errors (e.g., $N = 0.703 \pm 0.00294$) only indicate goodness of fit between data and a mathematical formula, not the error (more in Talk 14)
- Real errors have many components, from the experiment itself to the integrity of the material used and more....
- Calorimetry software fits a transform of the data which is the derivative of a conventional binding hyperbola. ITC data but can be transformed and fitted in other software
- Derivative plots tend to emphasise the information content in n and Kd but are firmly entrenched in the ITC literature.

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 $\Delta H$ in one experiment
The ‘meaning’ of $N = 0.7$

- ‘$N$’ is the ‘number of sites’, or ‘stoichiometry’ of the interaction.

- 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’.

- If not, ‘$N$’ can be viewed as an indicator of ‘active site concentration’ or an additional fitting parameter that allows concentration to float during fitting and thereby make the agreement with the data look better.

- In practice $N = 1 \pm 0.2$ is equivalent to $N \sim 1$. $N = 0.5$ and $N = 2$ etc., also have some potential physical meaning (e.g., dimer with one site etc., etc).

<table>
<thead>
<tr>
<th>NLLS Fitting values</th>
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| $N$             | 0.703  
| $K_a$           | 2.98E6  
| $K_d$           | 330 nM  
| $\Delta H$      | -6759  

High affinity binding

- As binding becomes tighter c-value ($[\text{cell}]/K_d$) becomes too large unless protein concentration is decreased.

- However, ($\text{cell concentration} \times \Delta H$) determines the total heat in the experiment and with current detection limits [protein] must be $>5-10$ uM unless $\Delta H$ is very large.

- This limits simple ITC measurements to $K_d$’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 $K_d$ 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)
- $\Delta H_{\text{obs}}$ competition $\sim = \Delta H_2 + (- \Delta H_1)$
- $K_{d_{\text{obs}}}$ competition $\sim = K_{d_1} \times K_{d_2}$
- KNI-764 inhibitor binding HIV-1 protease $K_d$ 32 pM

*Velazquez-Campoy et al 2001, Arch Biochem Biophys, 390, p169

Automated ITC

- ‘Higher’ throughput robotic instrument running $\sim 30$ titrations /day.
- Direct ligand screening, $K_d$ vs temp, buffer, pH etc.
- Indirect competition binding of established ligand in the presence of small molecule library. Both $\Delta H$ and $K_d$ are altered in “hits”. This screen is selective for ligands binding to the target site only.

$\Delta H_{\text{obs}}$ competition $\sim = \Delta H_2 + (- \Delta H_1)$
$K_{d_{\text{obs}}}$ competition $\sim = K_{d_1} \times K_{d_2}$
Weak binding

• As binding becomes weaker 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

No just Kd’s and ΔH
\( \Delta C_p \) of binding

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

\[ \Delta H = \int_0^T \Delta C_p \, dT \]
\[ \Delta C_p = \frac{\partial \Delta H}{\partial T} \]

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

\[ \Delta v = 0.39 \]

\( \Delta v \) (protonation change) of binding

- Titrations in different buffers indicates the net flux of protons.
- Differences in \( \Delta v \) 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, hetero-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 $k_{\text{obs}} (k_{\text{on}} + k_{\text{off}})$ becomes slower than the instrumental response the ITC peaks become broader
- Normally this occurs as the system ‘saturates’ and $k_{\text{on}}$ becomes slow due to limited free sites
- KinITC software*

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

Enzyme / Reaction Kinetics by ITC
A universal* method?

Hydrolysis of pNPP by PP1-g phosphatase

Rate = \left( \frac{1}{\Delta H} \right) \frac{dQ}{dt}

uM enzyme long duration complete turnover

nM enzyme short duration initial rate of turnover based on \( \Delta H \)

*Non-optical, no assay development or coupled reactions
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 stabilisation or destabilisation

- Mass action increases or decreases the stability as ligands bind to native or
Stability / Ligand screening using Tm

- CAP DSC ‘high’ throughput instrument (20-50 samples/day, 300 samples run unattended)
- Can evaluate binding parameters Kd, ΔH, ΔCp
- QPCR instrumentation has higher throughput and requires less material but uses a fluorescent reporter dye (Niesen et al., Nature Protocols 2007, 2, 2212).
- Prometheus instrument is equally quick and economic in material but uses changes in intrinsic fluorescence.
- Non-calorimetric methods measure ~ 50 samples /hr

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DSC and Complex mixtures

Normal human plasma n=15

Plasma from patients with a range of cancers and diseases

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 .... 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, mechanism
- Many other uses for ITC and DSC
- Enthalpy and Entropy are difficult to interpret in isolation since they include contributions from all interactions in the system.... Remember solvent!
Some examples of LMB work employing some calorimetry

**Ubiquitin Ser65 phosphorylation affects ubiquitin structure, chain assembly and hydrolysis.**

**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.**

**The hepatitis B virus preS1 domain hijacks host trafficking proteins by motif mimicry.**

**Bacterial actin MreB assembles in complex with cell shape protein RodZ.**