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Protein-carbohydrate interactions: Isothermal Titration Calorimetry

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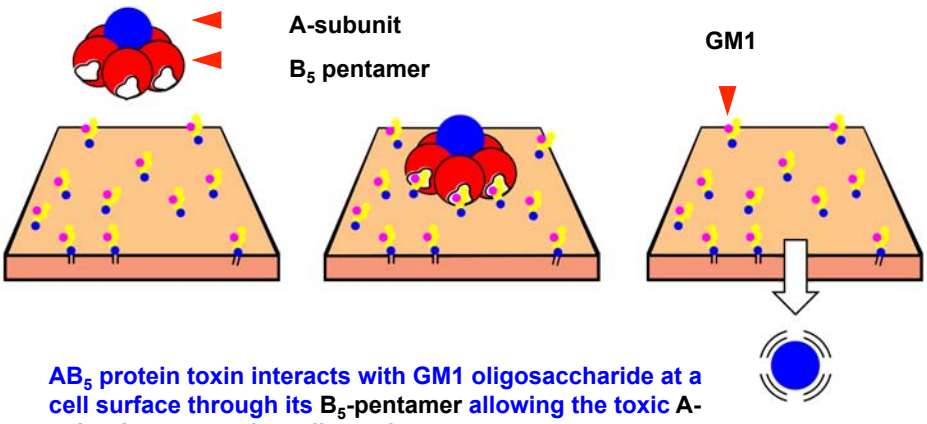
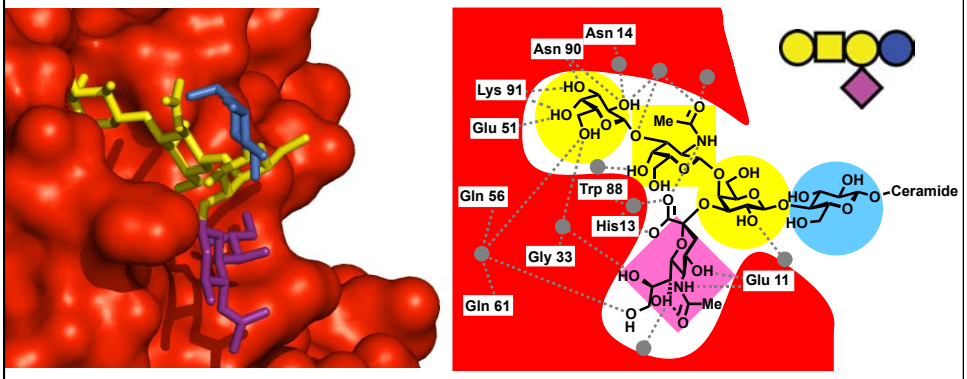
Cholera Toxin Invasion



AB_5 protein toxin interacts with GM1 oligosaccharide at a cell surface through its B_5-pentamer allowing the toxic A-subunit to cross the cell membrane.

The A subunit ADP ribosylates $G_{s\alpha}$ resulting in permanent activation of adenylate cyclase and overproduction of cyclic AMP, leading to loss of water into the gut and potentially fatal diarrhoea.

Crystal Structure of CTB-GM1os Complex



Branched oligosaccharide holds the protein in a "two fingered grip"

Extensive H-bonding between the three terminal residues and the protein

Remaining sugars point away from the protein – site of lipid attachment

E.A.Merritt, S. Sarfaty, F.van den Akker, C. L'Hoir, J.A. Martial, W.G.J.Hol, *Prot. Sci.* **1994**, *3*, 166-175;
E.A. Merritt, P. Kuhn, S. Sarfaty, J.L. Erbe, R.K. Holmes, W.G.J. Hol, *J. Mol. Biol.* **1998**, *282*, 1043-1059.

Receptor-ligand interaction

M **X** **MX**

$$K_a = \frac{[MX]}{[M][X]}$$

Units: L / mol

$$K_d = \frac{[M][X]}{[MX]}$$

Units: mol / L
i.e. K_d is a concentration

High affinity = large K_a, small K_d

Basic Thermodynamics…

M X MX

$$\Delta G^\circ = -RT \ln K_a$$

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

Free Energy Enthalpy Entropy

High affinity = large K_a, small K_d, large $-\Delta G^o$

Enthalpy

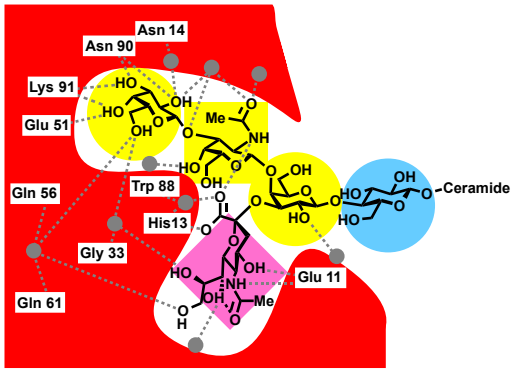
Changes in heat

Structure of the complex

- **Hydrogen bonding**
- **Van der Waals**

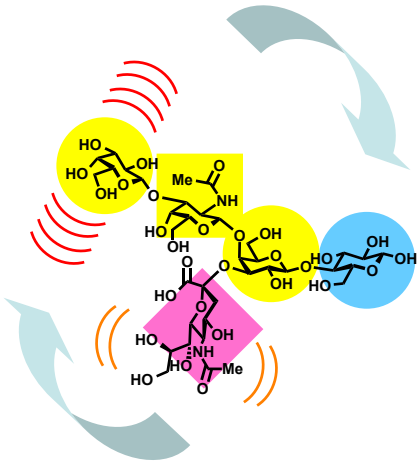
•Structure of the solvent

- **i.e. water**



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

Entropy

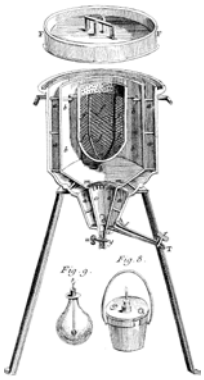


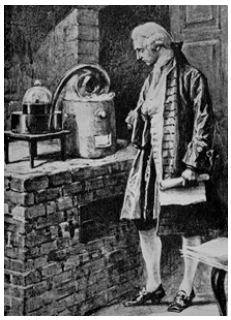
$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$$

Changes in disorder

- **Independent rotational and translational degrees of freedom**
  - **A complex is less disordered than two molecules**
- **Internal conformational dynamics**
  - **Flexible molecules lose entropy on binding**
- **Dynamics of the solvent**
  - **i.e. water**

Calorimetry – Measuring Heat





- Lavoisier and Laplace calorimeter to measure the element "caloric" in a sample of combustible oil (1784)

- oil burned in a lamp surrounded by ice
- heat determined by measuring amount of melted ice

Microcalorimetry

Differential Scanning Calorimetry

Solution heated/cooled from 10-100 °C

Used to measure unfolding temp and ΔH^{o} for DNA, proteins etc.

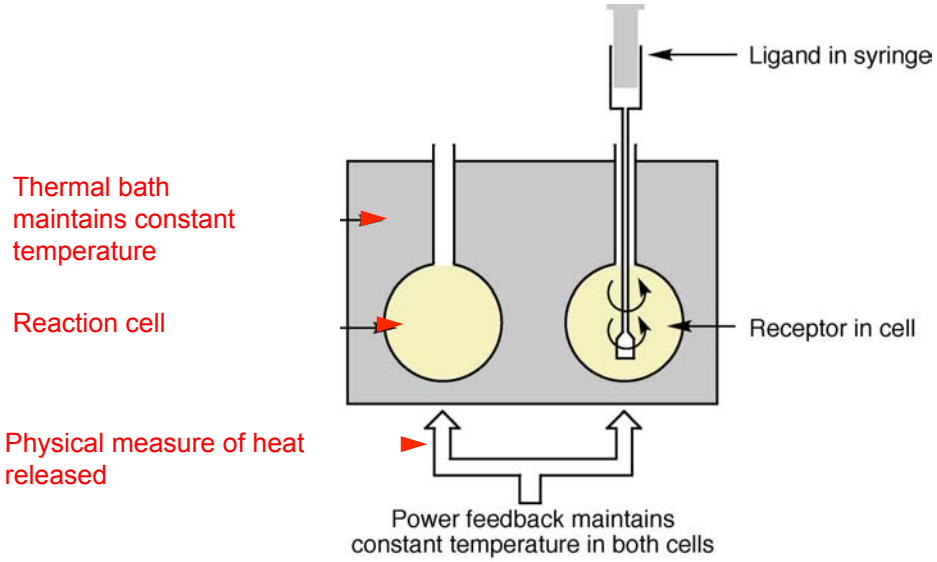
Isothermal Titration Calorimetry

Sample maintained at constant temp while two solutions are mixed

Used to measure

- protein-ligand interactions
- enzyme reactions
- ΔH^{o}

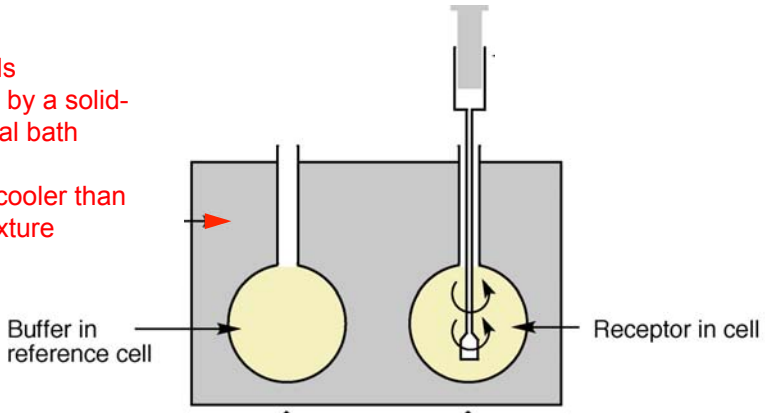
What's Inside an Isothermal Titration Calorimeter?



What's Inside an Isothermal Titration Calorimeter?

Sample cells surrounded by a solid-state thermal bath

• 1 degree cooler than reaction mixture



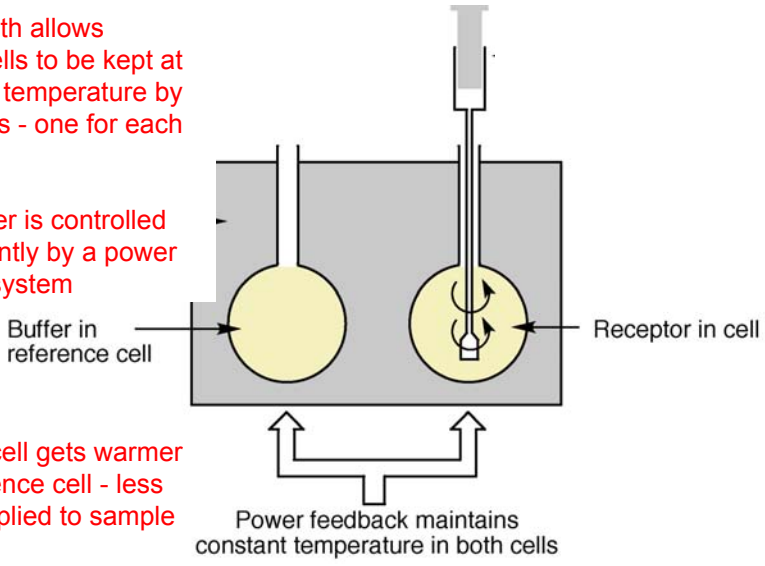
Two calorimeter cells

- the sample cell - usually contains the protein receptor solution
- the reference cell - usually contains buffer or water

What's Inside an Isothermal Titration Calorimeter?

Cooling bath allows reaction cells to be kept at a constant temperature by two heaters - one for each cell

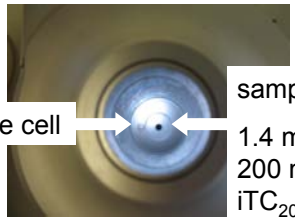
Each heater is controlled independently by a power feedback system



If sample cell gets warmer than reference cell - less power supplied to sample cell heater

Setting up the experiment (MicroCal VP-ITC)

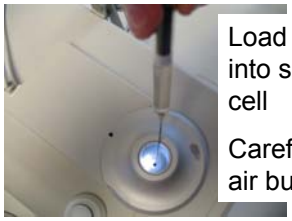
Load the sample cell and the syringe



reference cell

sample cell

1.4 mL or 200 mL for iTC_{200}



Load protein into sample cell

Careful! No air bubbles!

Suck ligand into syringe

Careful! Don't bend that needle!

Ready to go…

ITC just got smaller…

iTC_{200}

- Cell volume 200 mL
- Faster equilibration
- Faster titrations

at rest

sample loading

titration

washing

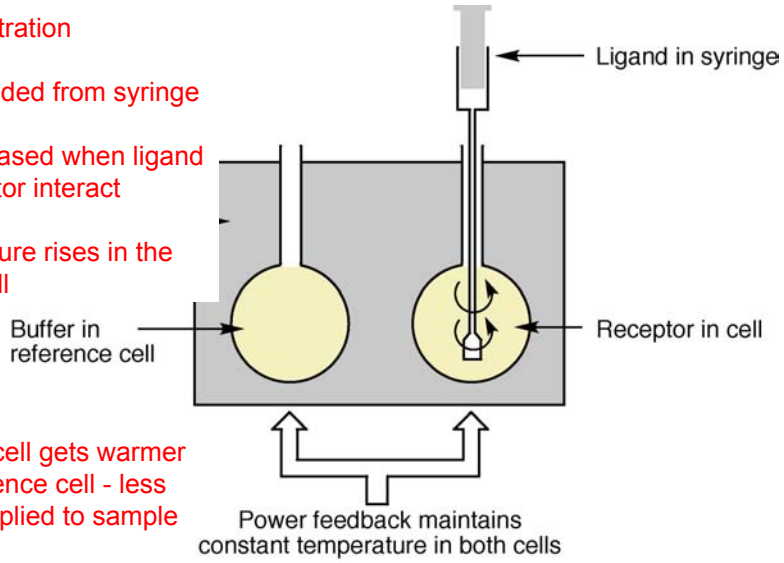
What's Inside an Isothermal Titration Calorimeter?

During a titration

• ligand added from syringe

• heat released when ligand and receptor interact

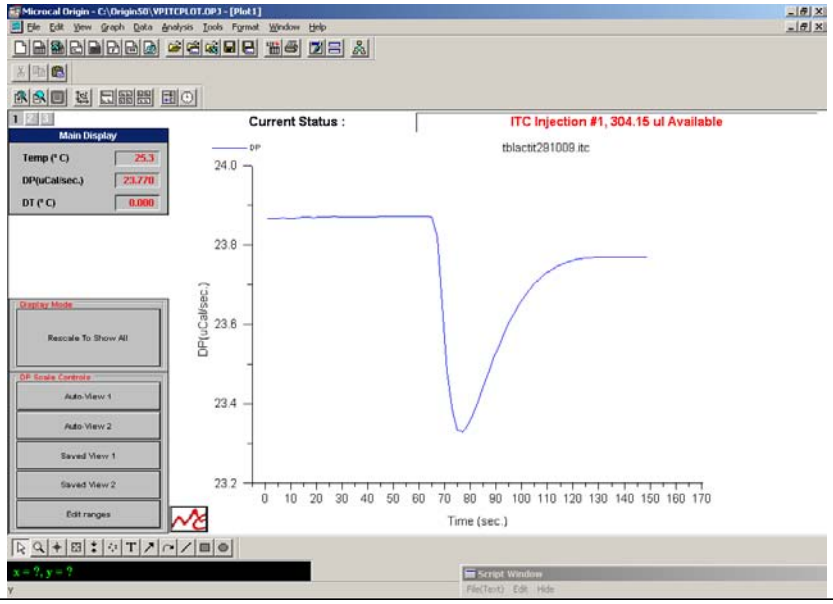
• temperature rises in the sample cell



If sample cell gets warmer than reference cell - less power supplied to sample cell heater

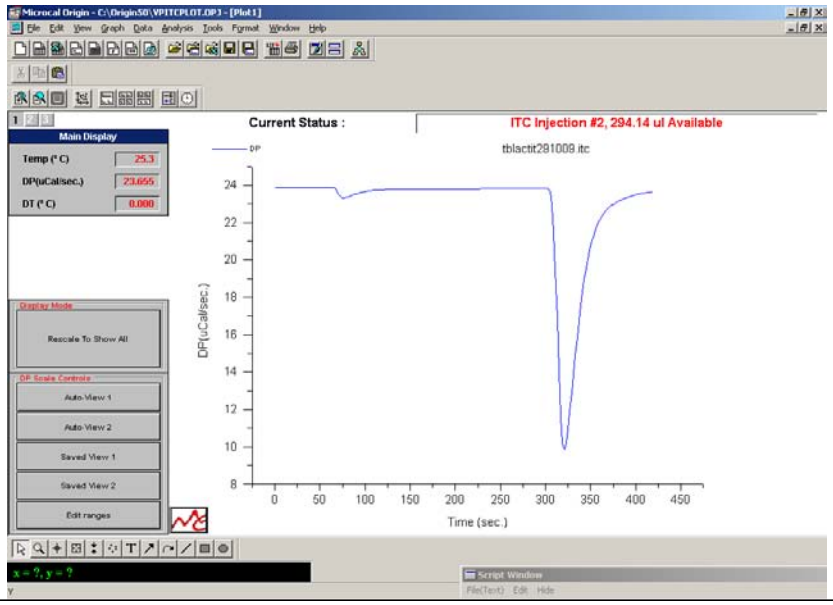
The first injection

A small throw-away injection as ligand diffuses into the cell during equilibration…



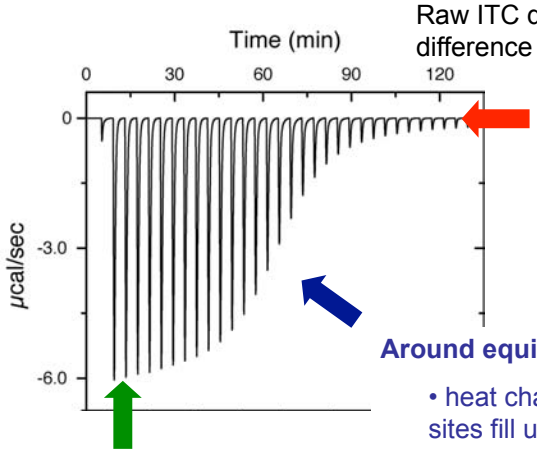
The second injection

Should be a lot bigger…



The Titration Data

Raw ITC data is a measure of the power difference supplied to each cell



End of titration

- all binding sites occupied – no further binding
- only "dilution peaks" on adding more ligand

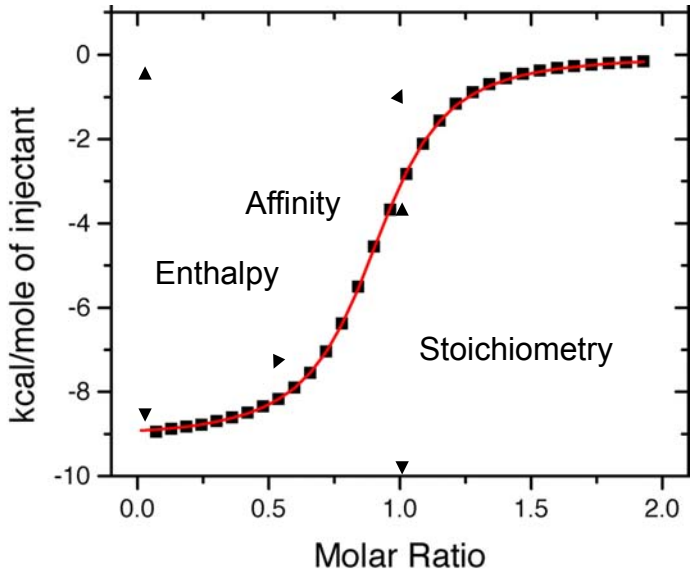
Around equivalence point

- heat change decreases as binding sites fill up

Start of titration

- large peaks – lots of complex formed on each injection
- equal height – virtually every ligand molecule becomes bound to receptor

How do we determine ΔH^o and ΔG^o from the curve?

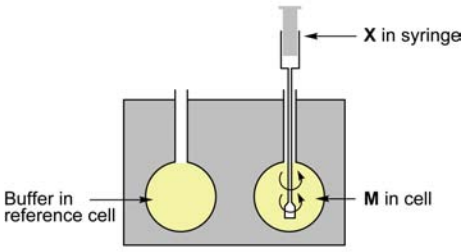


How do we determine ΔH^o and ΔG^o from the curve?

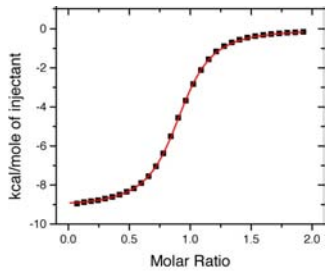
For 1:1 binding of ligand X and receptor M

$$X + M \rightleftharpoons MX$$

$$\frac{dQ}{d[X]_t} = \Delta H^{\circ} V_0 \left[\frac{1}{2} + \frac{1 - ([X]_t/[M]_t) - (K_d/[M]_t)}{2\sqrt{\left[1 + ([X]_t/[M]_t) + (K_d/[M]_t)\right]^2 - 4([X]_t/[M]_t)}} \right]$$



Isothermal Titration Calorimeter



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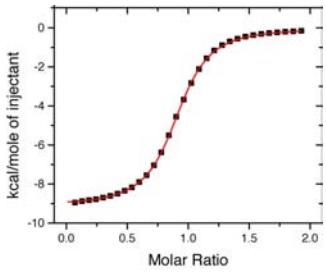
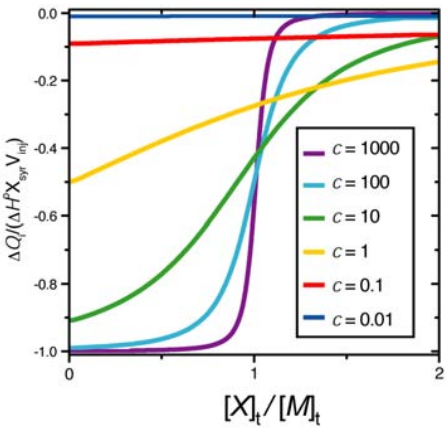
Shape of the curve depends on the value of c

$$c = \frac{1}{K_d/[M]_t} = \frac{[M]_t}{K_d} = K_a[M]_t$$



The curve shape depends on the "*c*-value"

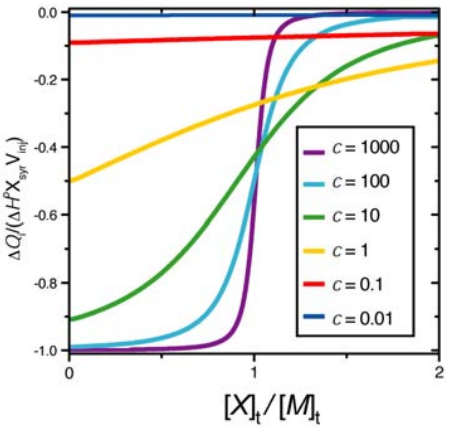


$$c = \frac{[M]}{K_d}$$

c > 10
sigmoidal curve that becomes steeper as c increases

c < 10
Curve becomes flatter

The curve shape depends on the "*c*-value"



$$c = \frac{[M]}{K_d}$$

c > 1000

$[M]_{total} >> K_d$

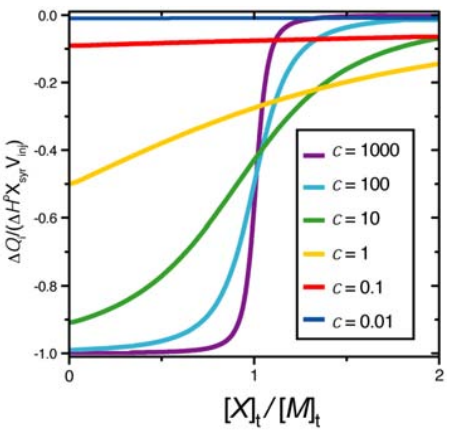
slope is too steep to determine K_d

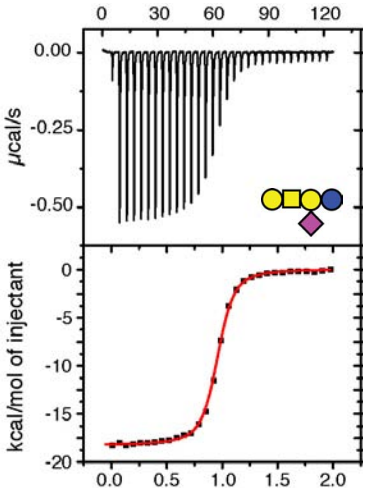
• only ΔH^o and *n* can be measured

For very high affinity ligands (low K_d) must use low receptor concentration

But low [M] gives very small signals… **K_d limit = 1 nM**

The curve shape depends on the "*c*-value"

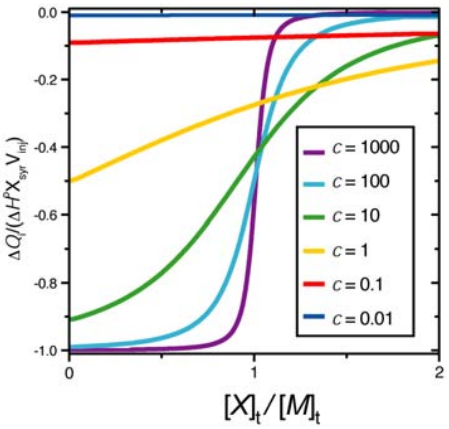




Cholera Toxin binds GM1os with K_d = 40 nM

If [CTB] = 10 μM then c = 250

The curve shape depends on the "*c*-value"



$$c = \frac{[\mathrm{M}]}{K_d}$$

c < 1

$[M]_{total} << K_d$

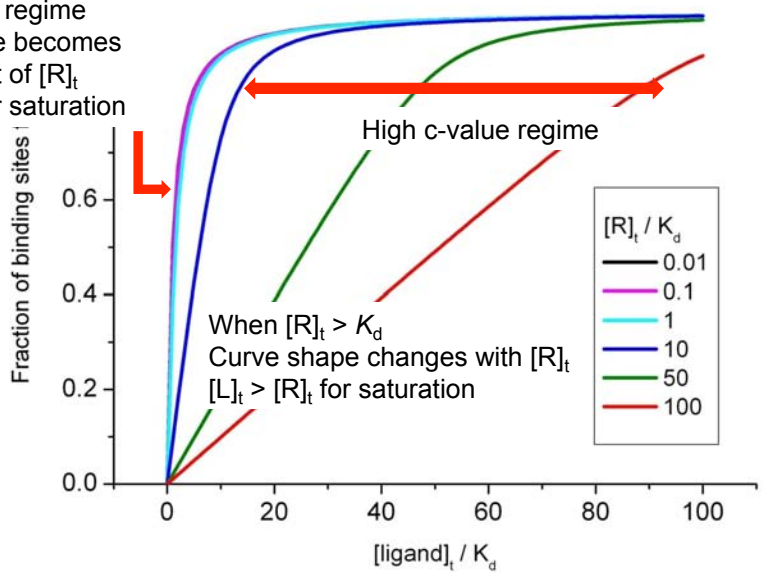
Curve becomes very flat

For very low affinity ligands (high K_d) must use high receptor concentration

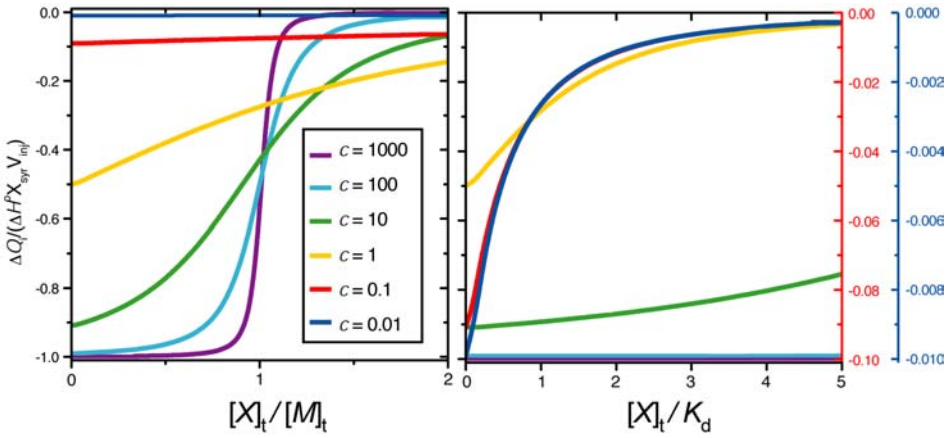
But proteins often soluble to only 1 mM… **K_d limit = 1 mM**

The Shape of the Binding Curve Changes if Receptor Concentration is Higher or Lower than K_d

Low c-value regime
Curve shape becomes independent of $[R]_t$
$[L]_t >> K_d$ for saturation



Alternative Depiction of the ITC binding Isotherm

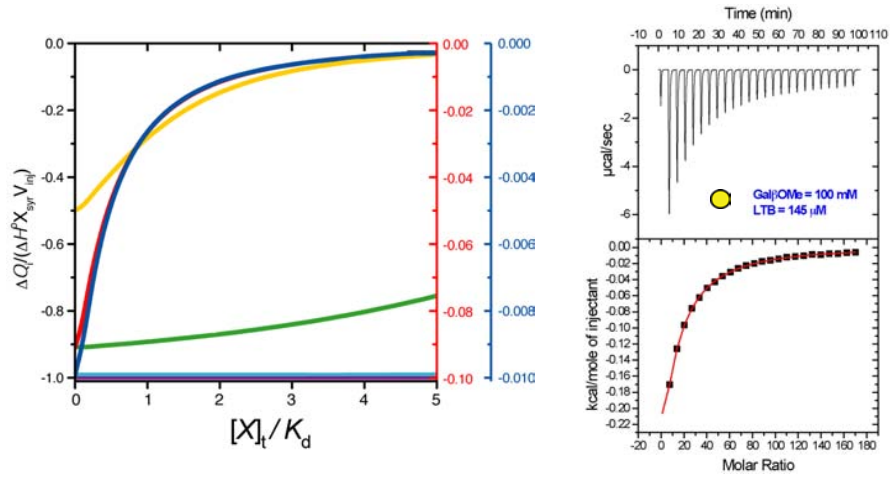


For very low affinity ligands (high K_d) can use low c-value titrations

But must add many equivalents of ligand… **K_d limit = 50 mM?**

W. B. Turnbull and A. H. Daranas, *J. Am. Chem. Soc.* **2003**, *125*, 14859-14866

"*c*-value" curve with heat vs. ligand to K_d ratio

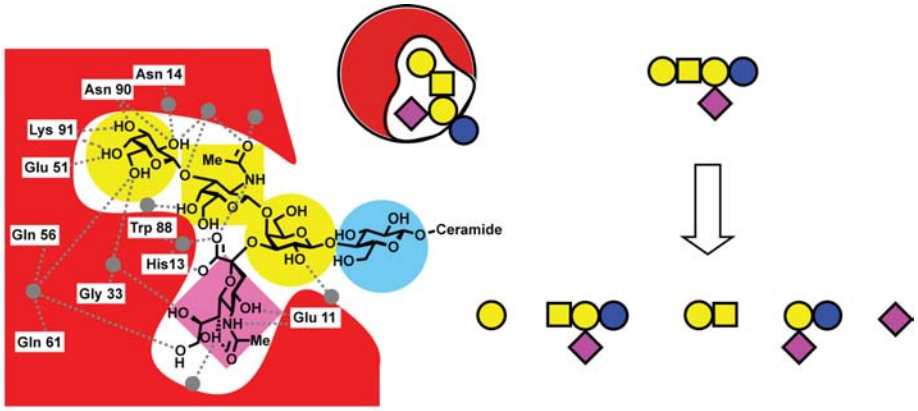


ΔH^o and K_d can still be determined but not stoichiometry

Must know concentrations accurately

Cholera Toxin binds GalβOMe with K_d = 15 mM [CTB] = 145 µM c = 0.01

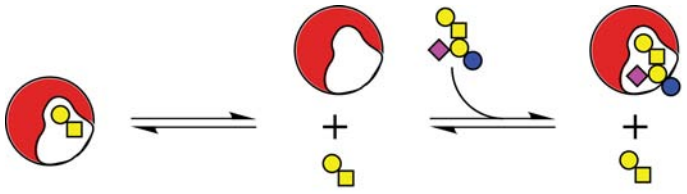
Dissecting the GM1–CTB Interaction



Objective: to evaluate the contribution that each monosaccharide makes to the CTB—GM1 interaction in solution.

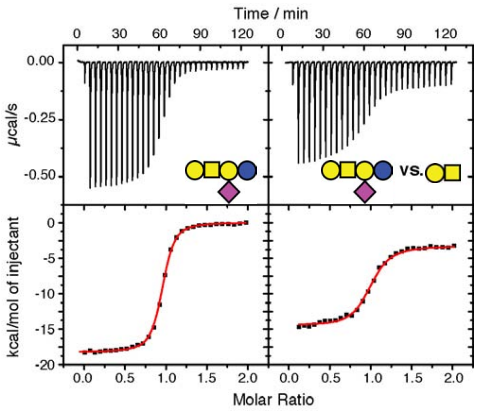
Disconnect oligosaccharide into fragments and measure each interaction with CTB

Very high and very low affinity systems can be studied using competition titrations



- High affinity ligand added to a solution of the low affinity complex
- High affinity ligand displaces the low affinity ligand
- Change in the apparent affinity and apparent enthalpy
- If parameters for one ligand are known, possible to calculate for the other ligand

Example Displacement Titrations



10 µM CTB

110 µM

0 or 25 mM

high affinity ligand

high affinity ligand plus a lower affinity ligand

Very steep curve for high affinity ligand becomes more gentle in the presence of a lower affinity competing ligand

Summary of ITC Results

Ligand	K_d	ΔG° calmol^{-1}	ΔH° calmol^{-1}	TΔS° calmol^{-1}	n
	43.3 ± 1.4 **nM**	**-10,040** ± 20	**-17,450** ± 30	**-7,450** ± 30	**1.00**
	14.8 ± 1.6 **mM**	**-2,500** ± 70	**-9,020** ± 480	**-6,530** ± 480	**0.94**
	2.0 ± 0.2 **mM**	**-3,670** ± 90	**-4,350** ± 480	**-690** ± 480	**0.99**
	7.6 ± 0.8 **mM**	**-2,890** ± 80	**-10,150** ± 430	**-7,270** ± 450	**1.06**
	0.21 ± 0.1 **M**	**-920** ± 280	**-10,700** ± 8,600	**-9,770** ±8340	**1.06**

GM1os pentasaccharide very high affinity

All fragments very low affinity

W. B. Turnbull, B. L. Precious, S. W. Homans, *J. Am. Chem. Soc.* **2004**, *126*, 1047-1054

Summary of ITC Results

Ligand	K_d	ΔG° calmol^{-1}	ΔH° calmol^{-1}	TΔS° calmol^{-1}	n
	**43.3** ± 1.4 **nM**	**-10,040** ± 20	**-17,450** ± 30	**-7,450** ± 30	**1.00**

Big increase in affinity from Gal-GalNAc disaccharide to GM1 pentasaccharide

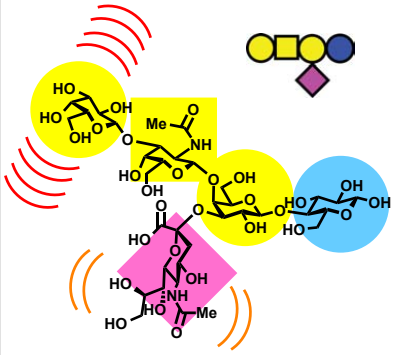
	**7.6** ± 0.8 **mM**	**-2,890** ± 80	**-10,150** ± 430	**-7,270** ± 450	**1.06**

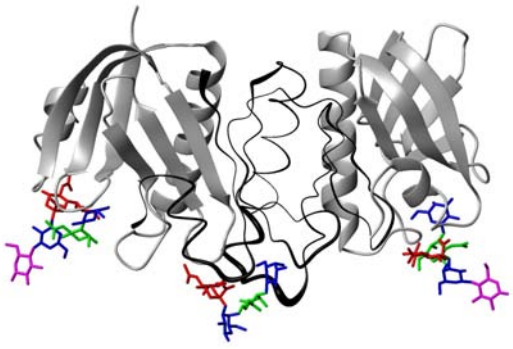
However, very similar TΔS° for the two ligands.

Contribution of sialic acid is totally enthaplic

Implies extra interactions with no loss of conformational entropy

Change in Conformational Entropy on Binding





Terminal Gal-GalNAc linkage is more flexible than Sia-Gal linkage

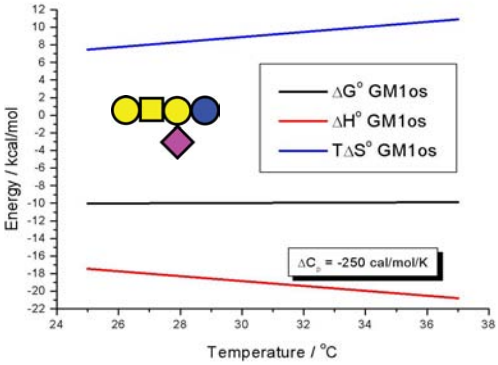
- Greatest loss of conformational entropy for Gal binding

Middle subunit as a sausage depiction – the width of the sausage indicates how much the backbone atoms move on binding

- Tightening of loop around galactose on binding

Warning! Be careful how you interpret ΔH^o!

ΔH^o and $T\Delta S^o$ change with temperature: ΔC_p



$$\Delta C_p = \frac{\Delta H_2^o - \Delta H_1^o}{T_2 - T_1}$$

$$\Delta C_p = \frac{T_2 \Delta S_2^o - T_1 \Delta S_1^o}{T_2 - T_1}$$

Depends on ΔC_p – the change in specific heat capacity on binding

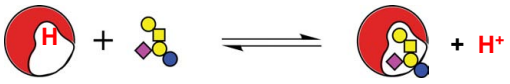
– ability of the system to absorb heat

$T\Delta S^o$ also dependent on ΔC_p – Entropy-Enthalpy Compensation

ΔG^o is essentially independent of temperature

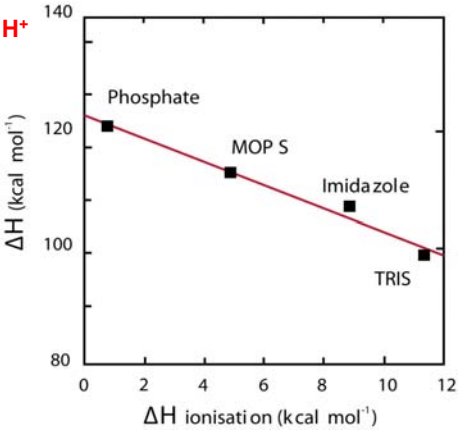
ΔH^o can also be affected by coupled reactions e.g., proton transfer

$$\Delta H_{observed} = \Delta H_{interaction} + \Delta H_{proton\ transfer}$$



Ligand binding sometimes coupled to proton transfer to or from the protein…

-size of $\Delta H_{proton\ transfer}$ depends on the buffer ionisation enthalpy
- must repeat titration in several different buffers



Summary

ITC is a useful technique for studying many concentration-dependent solution phenomena

It is always preferable to have a sigmoidal curve

- $10 < c < 500$

However low affinity systems can be studied as low c-value curves

Low and high affinity systems can also be studied by competition titrations

Beware the effects of coupled reactions and ΔC_p when interpreting ΔH^o