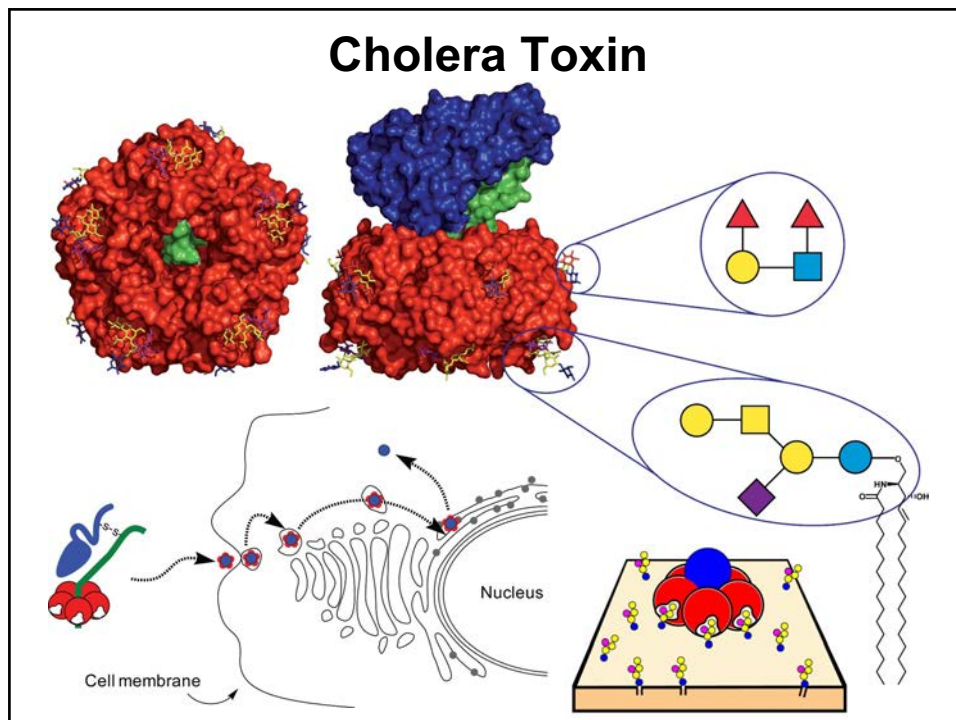


Protein-carbohydrate interactions: Isothermal Titration Calorimetry

Professor Bruce Turnbull

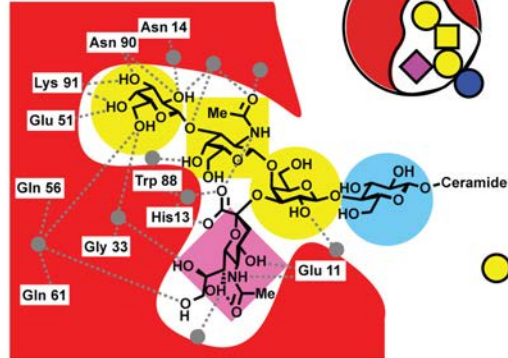
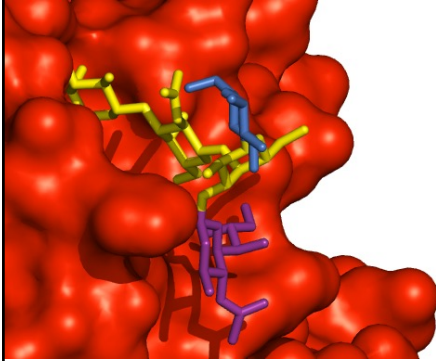
School of Chemistry and
Astbury Centre for Structural Molecular Biology
University of Leeds

1



2

Structure of CTB-GM1os Complex

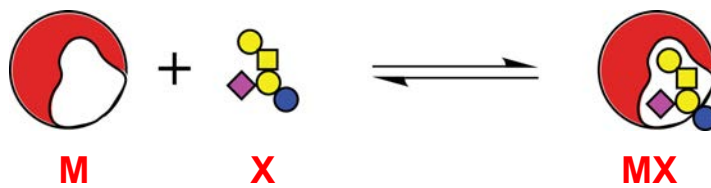


Branched oligosaccharide holds the protein in a “two fingered grip”
 Extensive H-bonding between the three terminal residues and the protein
 Remaining sugars point away from the protein – site of lipid attachment

E.A. Merritt, P. Kuhn, S. Sarfaty, J.L. Erbe, R.K. Holmes, W.G.J. Hol, *J. Mol. Biol.* **1998**, 282, 1043-1059.

3

Receptor-ligand interaction



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

Units: L / mol

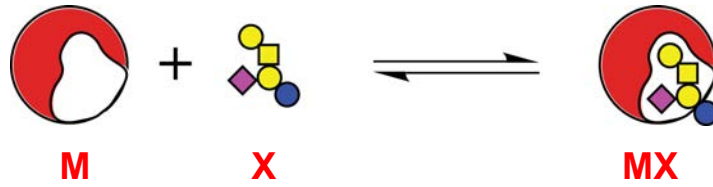
$$K_d = \frac{[M][X]}{[MX]}$$

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

High affinity = large K_a , small K_d

4

Basic Thermodynamics...



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

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

Free Energy Enthalpy Entropy

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

5

Enthalpy

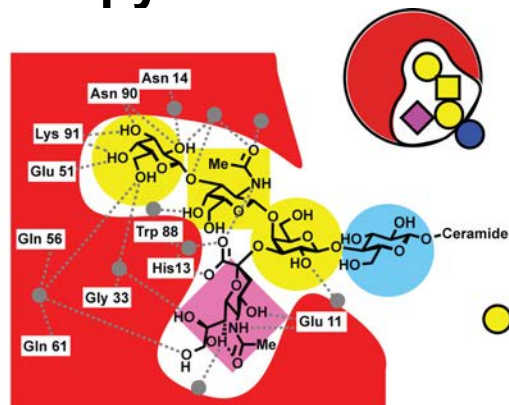
Changes in heat

Structure of the complex

- Hydrogen bonding
- Van der Waals

•Structure of the solvent

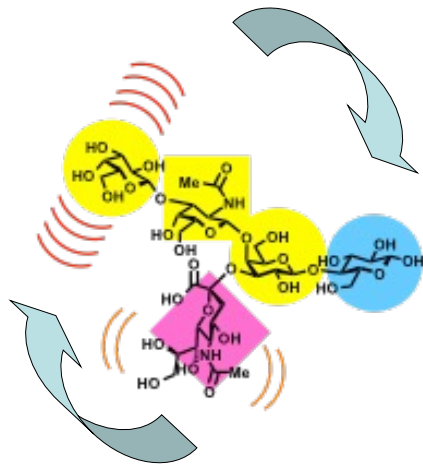
- i.e. water



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

6

Entropy



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

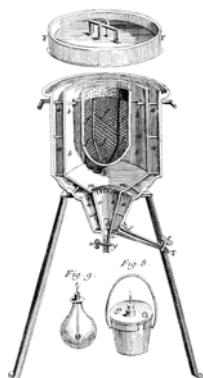
- **Dynamics of the solvent**

• i.e. water

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

7

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

8

Microcalorimetry

Differential Scanning Calorimetry

- Solution heated/cooled from 10-100 °C
- Used to measure unfolding temp and ΔH°



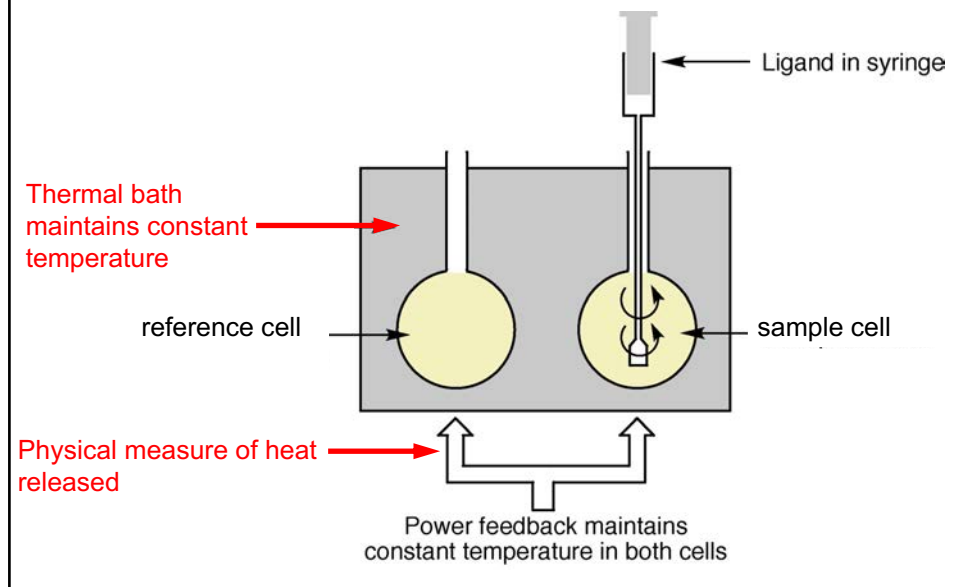
Isothermal Titration Calorimetry

- Sample maintained at constant temp while two solutions are mixed
- Used to measure
 - protein-ligand interactions
 - enzyme reactions
 - ΔH°



9

What's Inside an Isothermal Titration Calorimeter?



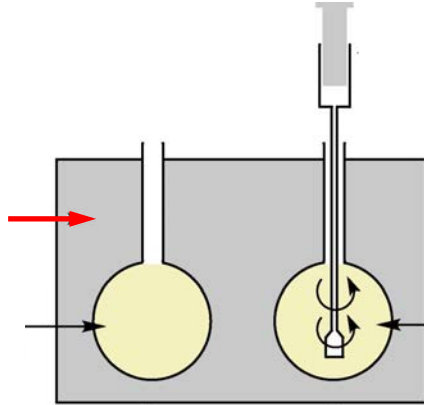
10

What's Inside an Isothermal Titration Calorimeter?

Sample cells surrounded by a solid-state thermal bath

- 1 degree cooler than reaction mixture

reference cell



sample cell

Two calorimeter cells

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



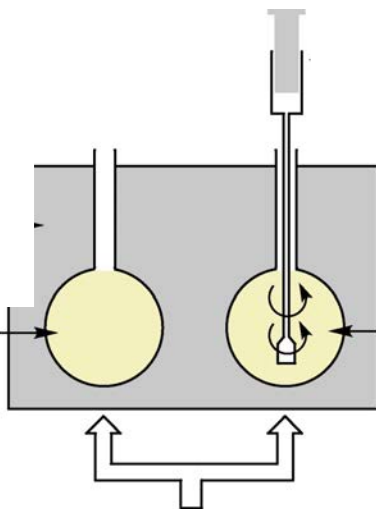
11

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

reference cell



sample cell

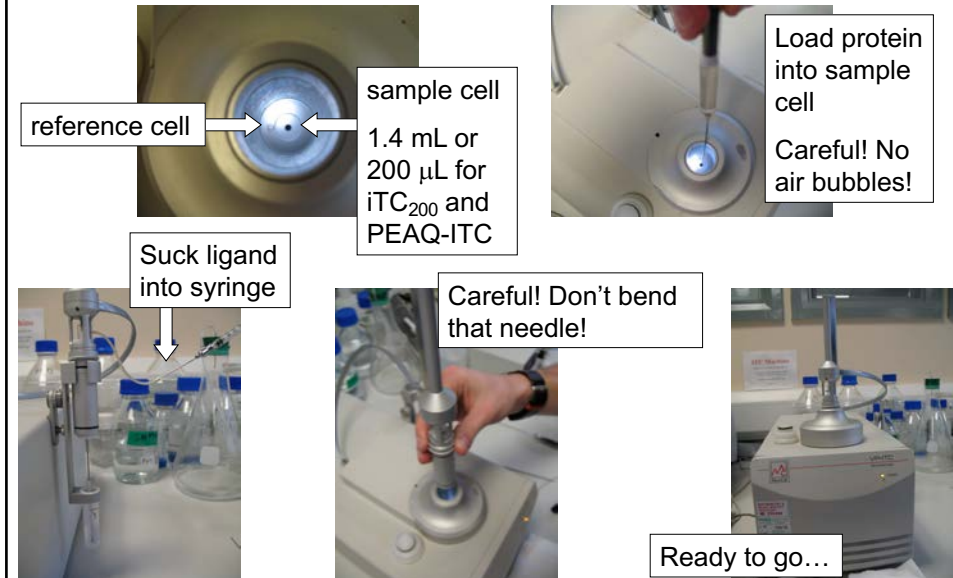
If sample cell gets warmer than reference cell - less power supplied to sample cell heater

Power feedback maintains constant temperature in both cells

12

Setting up the experiment (MicroCal VP-ITC)

Load the sample cell and the syringe



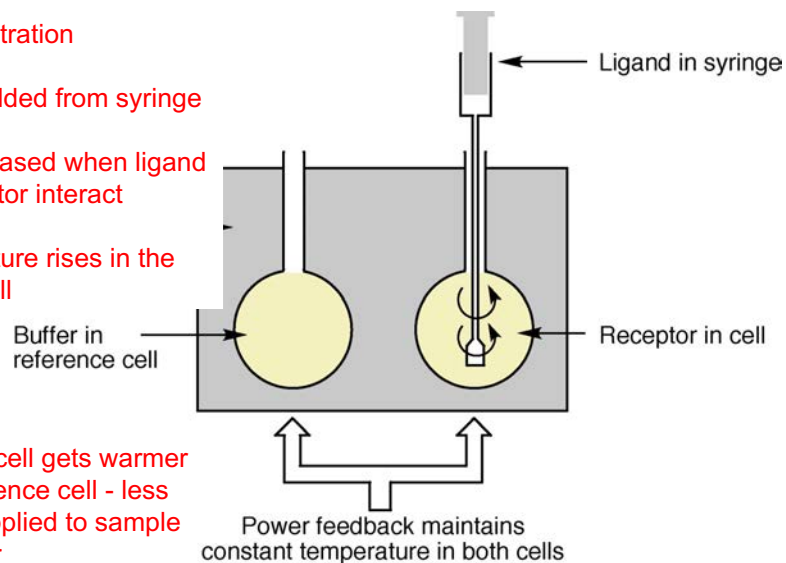
13

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

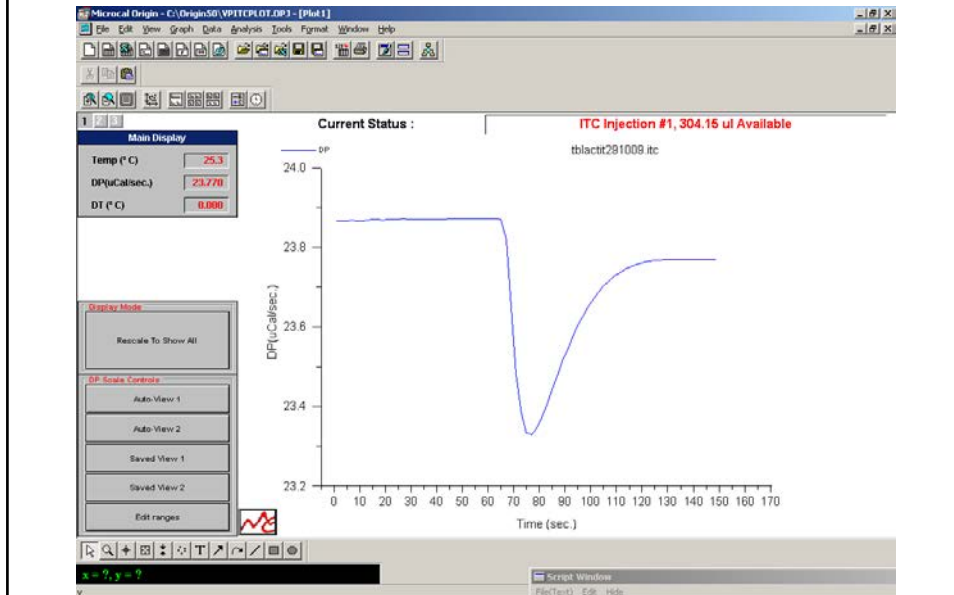
If sample cell gets warmer than reference cell - less power supplied to sample cell heater



14

The first injection

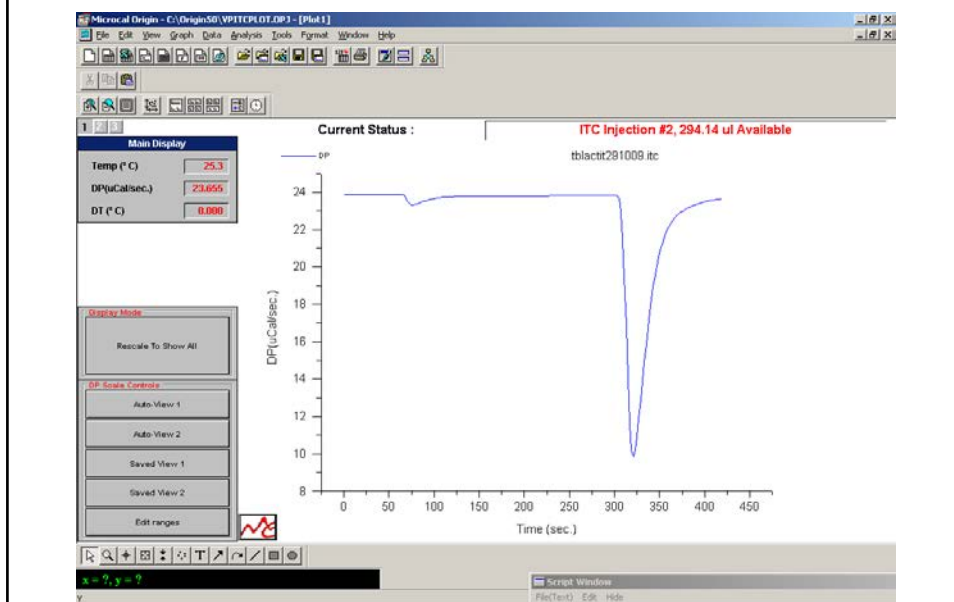
A small throw-away injection as ligand diffuses into the cell during equilibration...



15

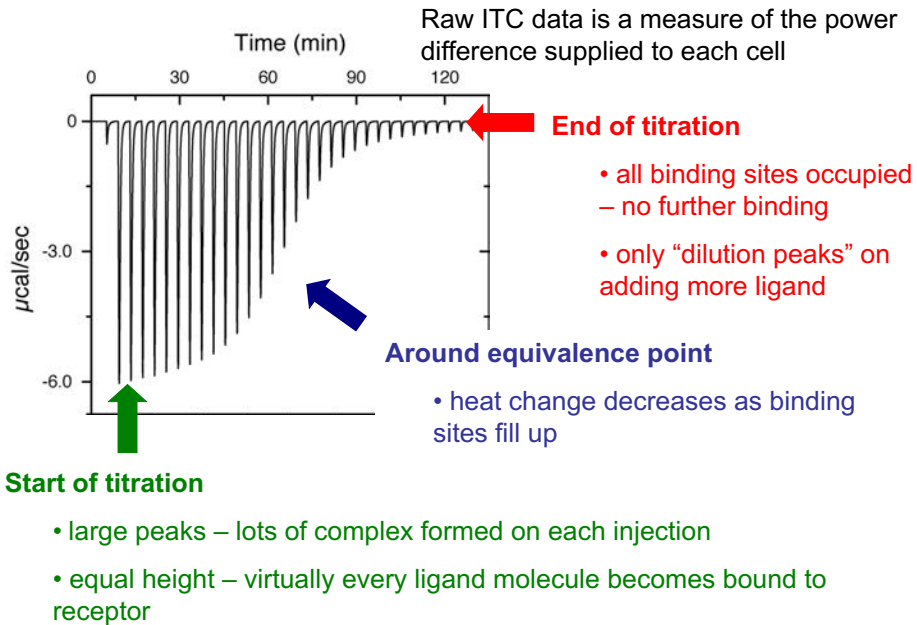
The second injection

Should be a lot bigger...



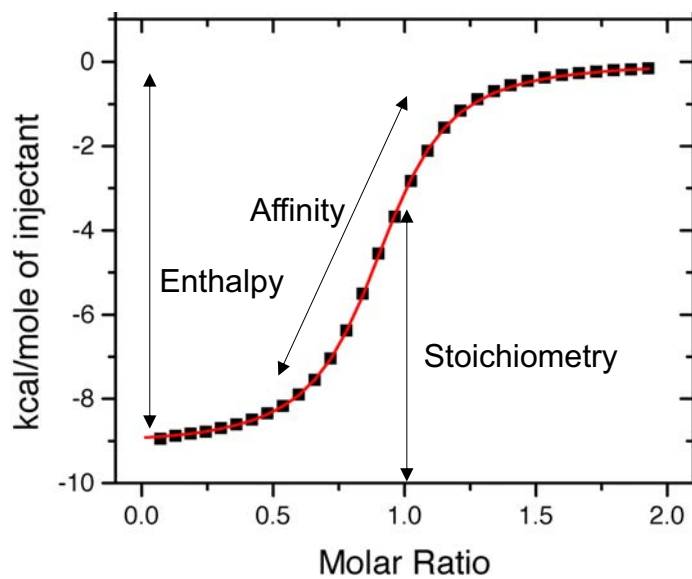
16

The Titration Data



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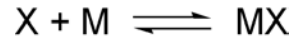
How do we determine ΔH° and ΔG° from the curve?



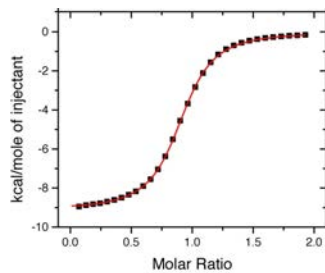
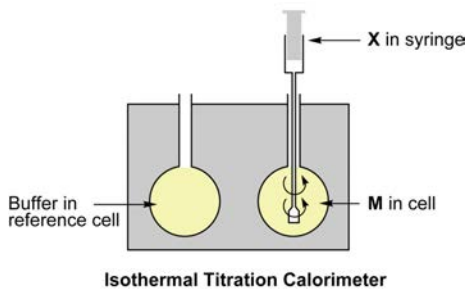
18

How do we determine ΔH° and ΔG° from the curve?

For 1:1 binding of ligand X and receptor M



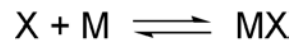
$$\frac{dQ}{d[X]_t} = \Delta H^\circ V_0 \left[\frac{1}{2} + \frac{1 - ([X]_t/[M]_t) - (K_d/[M]_t)}{2\sqrt{[1 + ([X]_t/[M]_t) + (K_d/[M]_t)]^2 - 4([X]_t/[M]_t)}} \right]$$



19

How do we determine ΔH° and ΔG° from the curve?

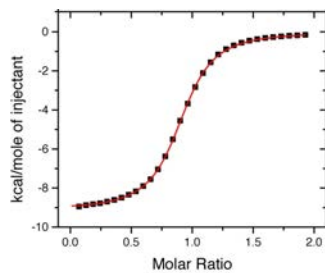
For 1:1 binding of ligand X and receptor M



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

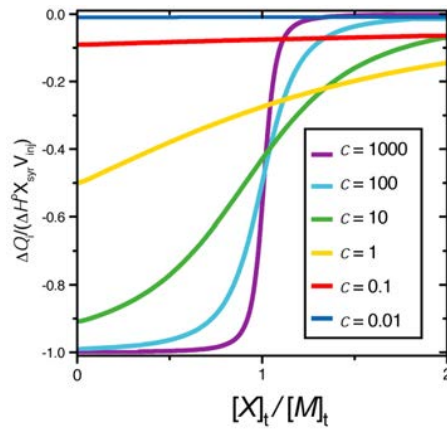
Shape of the curve depends on the value of c

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



20

The curve shape depends on the “c-value”



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

c > 10

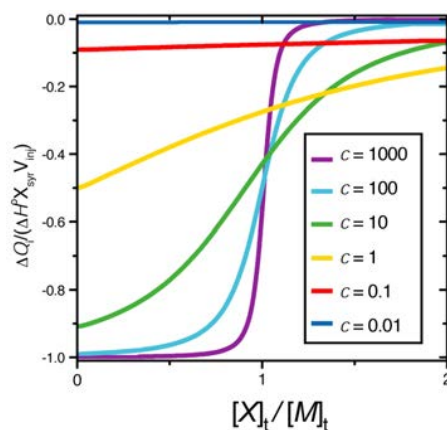
sigmoidal curve that becomes steeper as c increases

c < 10

Curve becomes flatter

21

The curve shape depends on the “c-value”



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

c > 1000

$[M]_{\text{total}} \gg K_d$

slope is too steep to determine K_d

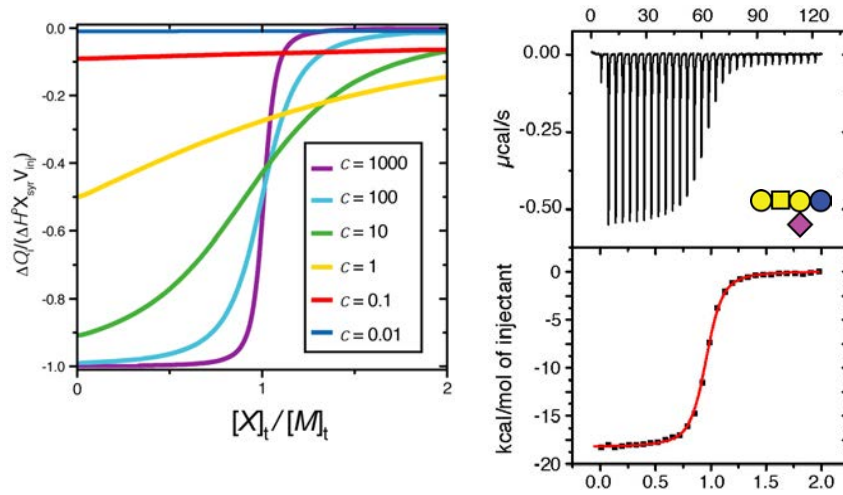
• only ΔH° and n can be measured

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

22

The curve shape depends on the “c-value”

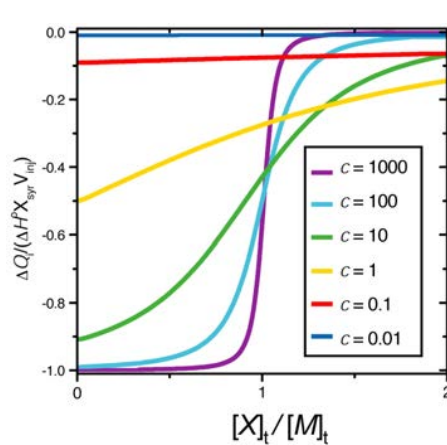


Cholera Toxin binds GM1os with $K_d = 40 \text{ nM}$

If $[\text{CTB}] = 10 \mu\text{M}$ then $c = 250$

23

The curve shape depends on the “c-value”



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

$c < 1$

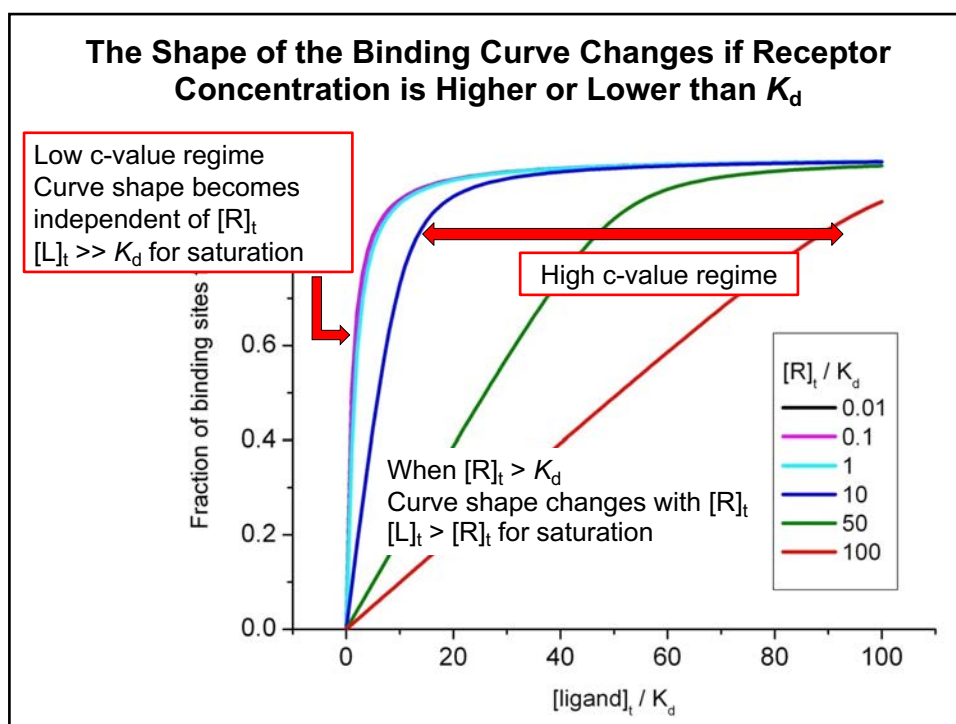
$[M]_{\text{total}} \ll K_d$

Curve becomes very flat

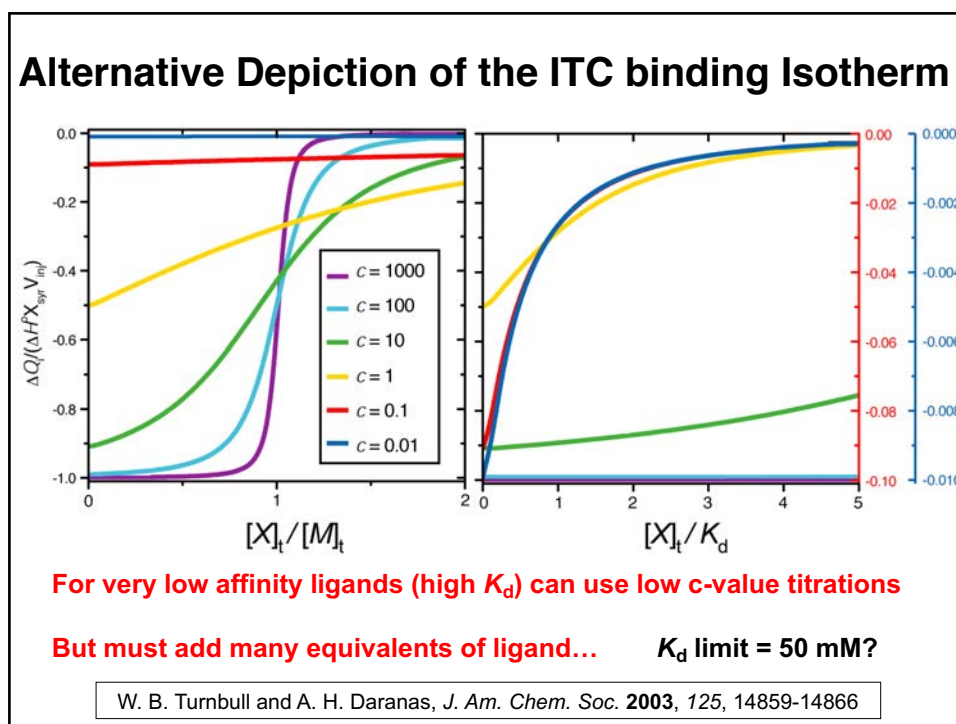
For very low affinity ligands (high K_d) must use high receptor concentration

But proteins often soluble to only 1 mM... K_d limit = 1 mM

24

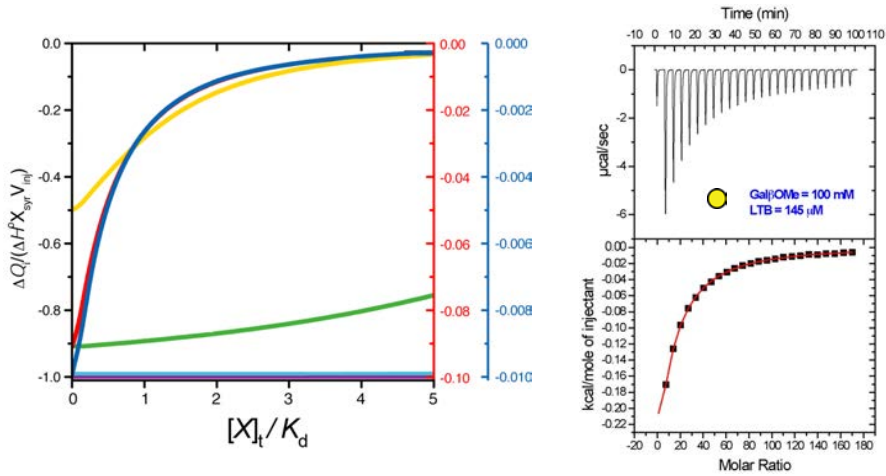


25



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“c-value” curve with heat vs. ligand to K_d ratio



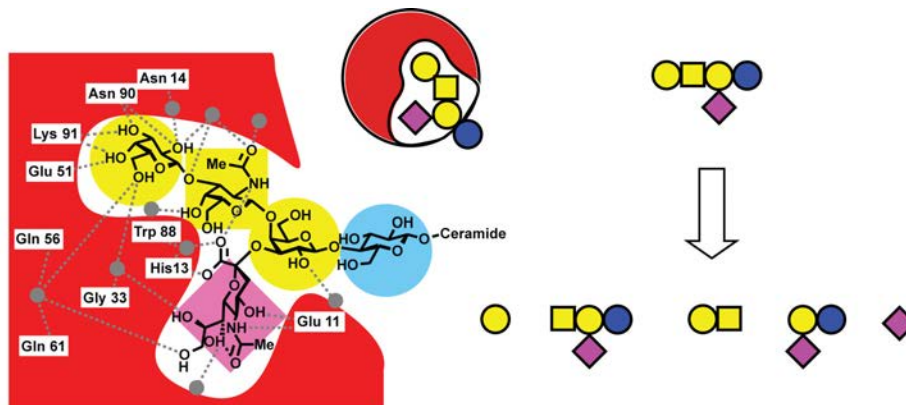
ΔH° and K_d can still be determined but not stoichiometry

Must know concentrations accurately

Cholera Toxin binds Gal β OMe with $K_d = 15 \text{ mM}$ [CTB] = $145 \text{ }\mu\text{M}$ $c = 0.01$

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Dissecting the GM1–CTB Interaction

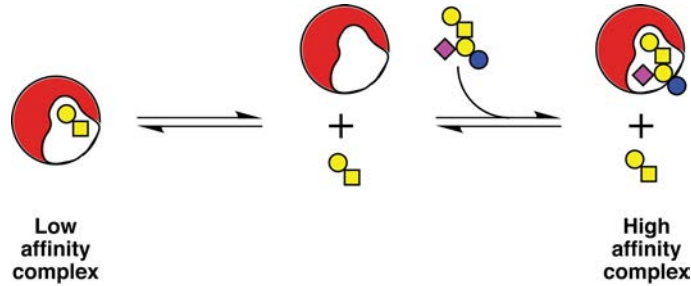


Objective: to evaluate the contribution that each monosaccharide makes to the CTB–GM1 interaction in solution.

Disconnect oligosaccharide into fragments and measure each interaction with CTB

28

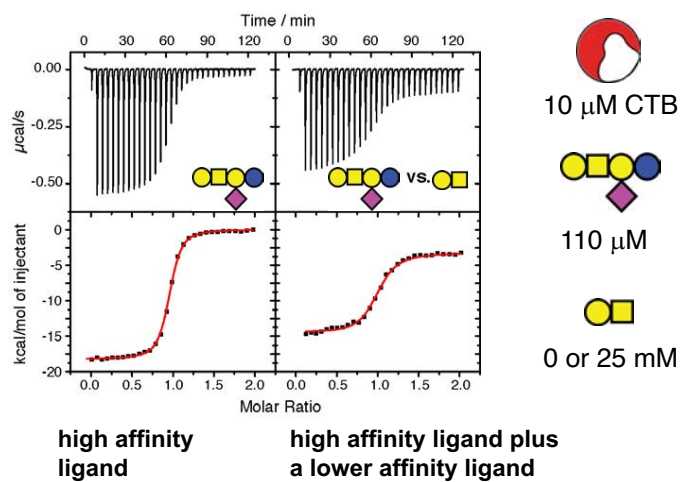
Very high and very low affinity systems can be studied using competition titrations



- 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

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Example Displacement Titrations




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

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Summary of ITC Results

Ligand	K_d	ΔG° calmol ⁻¹	ΔH° calmol ⁻¹	$T\Delta S^\circ$ 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

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Summary of ITC Results

Ligand	K_d	ΔG° calmol ⁻¹	ΔH° calmol ⁻¹	$T\Delta S^\circ$ 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
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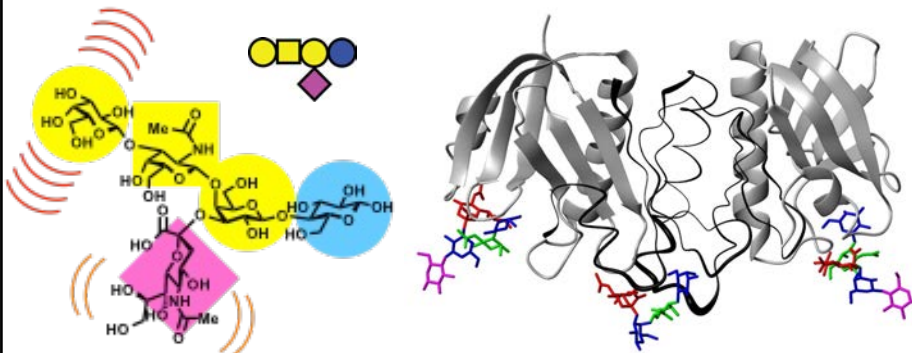
However, very similar $T\Delta S^\circ$ for the two ligands.

Contribution of sialic acid is totally enthalpic

Implies extra interactions with no loss of conformational entropy

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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

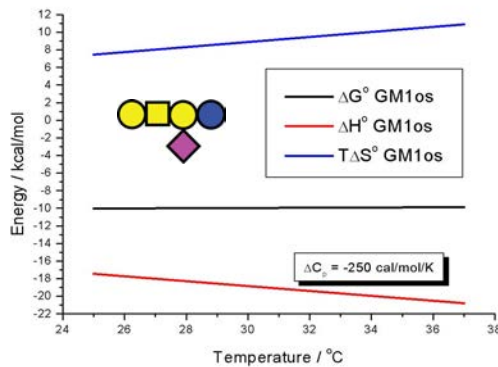
33

Warning! Be careful how you interpret ΔH° !



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



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

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

Depends on ΔC_p – the change in specific heat capacity on binding
 – ability of the system to absorb heat

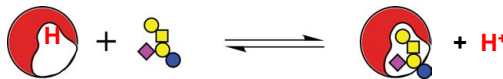
$T\Delta S^\circ$ also dependent on ΔC_p – Entropy-Enthalpy Compensation

ΔG° is essentially independent of temperature

35

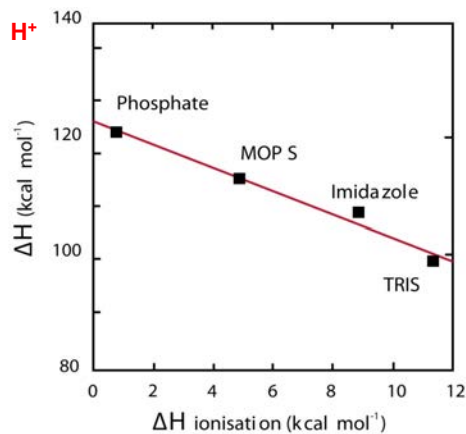
ΔH° can also be affected by coupled reactions e.g., proton transfer

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



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

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



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Summary

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

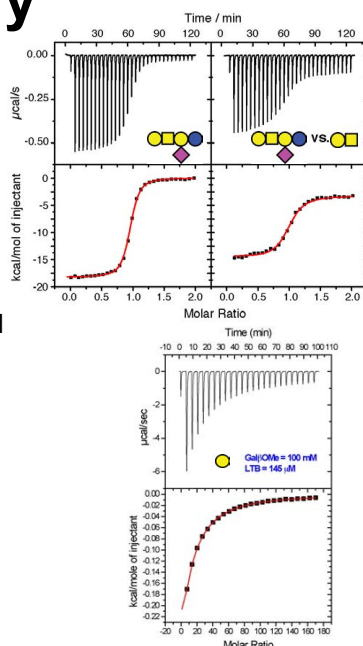
It is always preferable to have a sigmoidal curve

$$\bullet 10 < c < 500$$

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

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

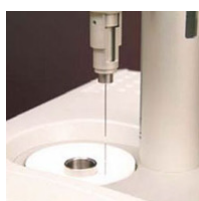
Beware the effects of coupled reactions and ΔC_p when interpreting ΔH^0



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This afternoon – demonstration of iTC₂₀₀ or PEAQ-ITC and ITC data analysis

- Cell volume 200 mL
- Faster equilibration
- Faster titrations



at rest



sample loading



titration



washing

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