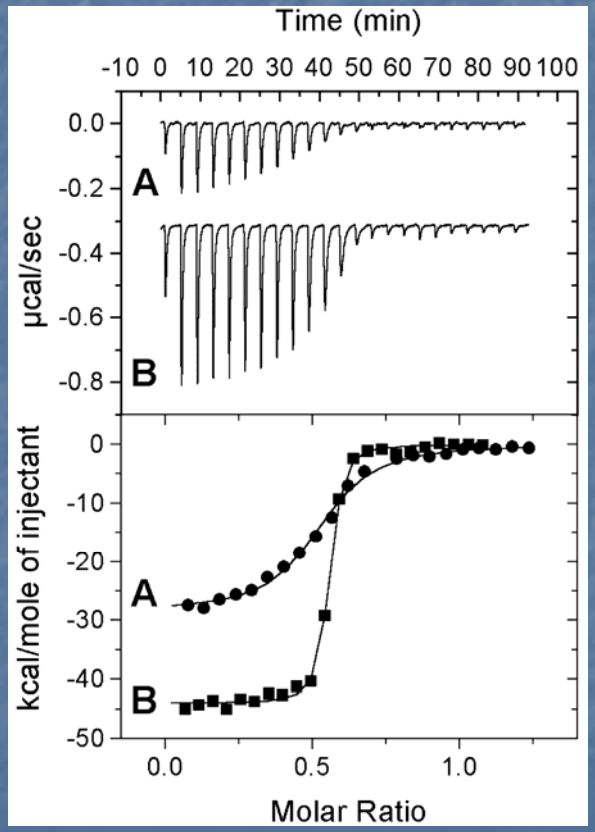
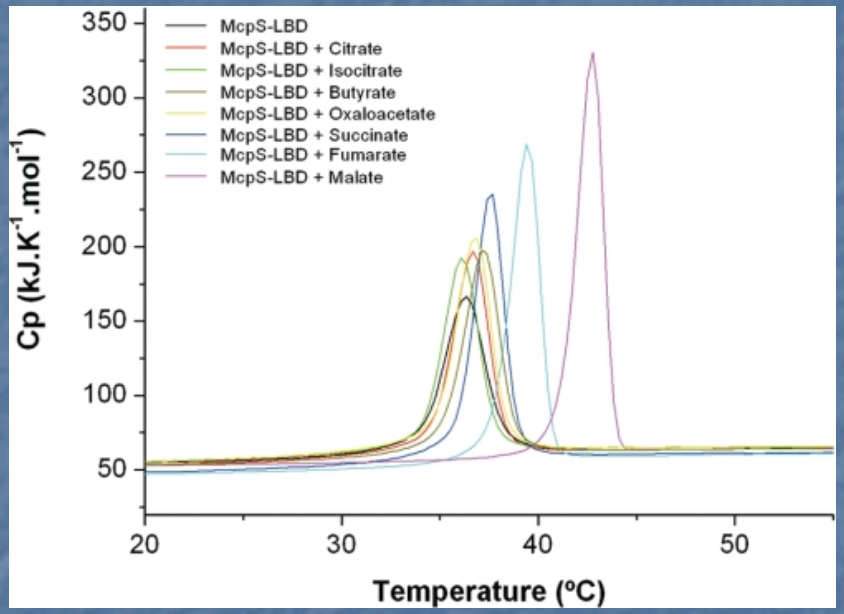
The use of microcalorimetric techniques to tackle diverse questions in life science





ITC

DSC

Presentations can be downloaded from

http://krell-laboratory.com/

Aims of the course

- Principle of analysis and instrumentation
- Information obtained and questions that can be answered
- Experimental design and optimisation of microcalorimetric analyses
- Data analysis
- Assessment of the quality of microcalorimetric data published

Thermodynamics of molecular interaction: what makes that there is binding?

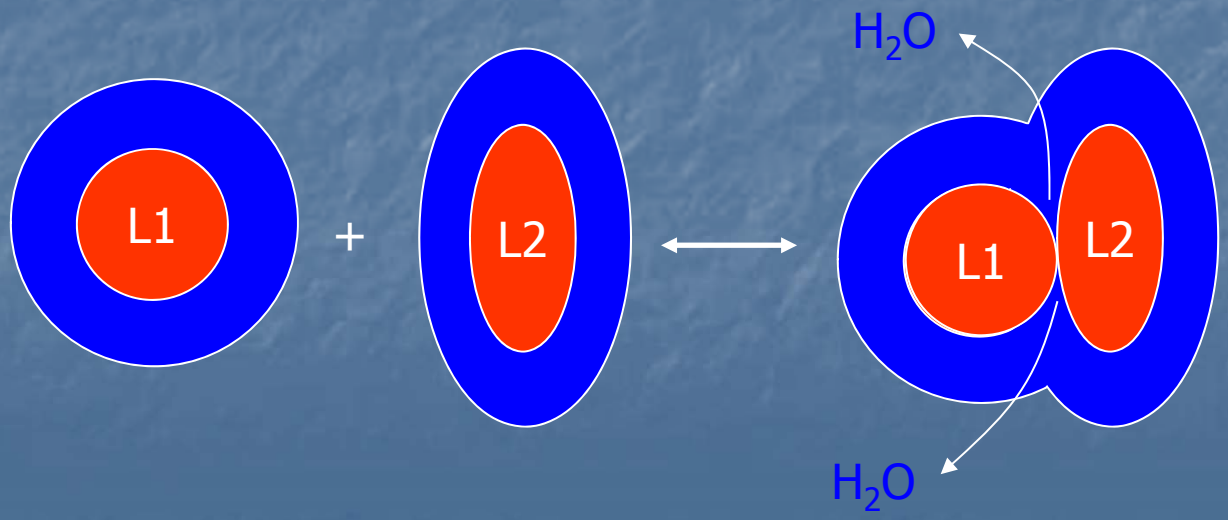
- Only if ΔG is negative, there is binding

$$\Delta G = - RT \ln K_A = \Delta H - T\Delta S$$

R = gas constant
T = absolute temperature

G = free energy
H = enthalpy
S = entropy
K_A = association constant
K_D = dissociation constant, $K_D = 1/K_A$

K_D = concentration of which half the ligand is bound, half is free

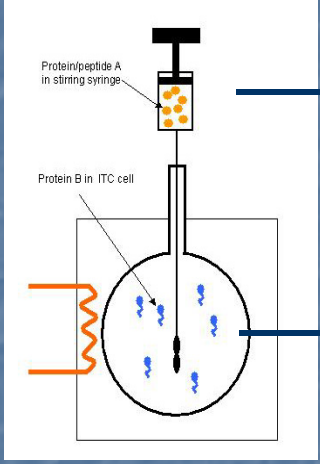


$$\Delta H = \Delta H_{for} + \Delta H_{rup}$$

$$\Delta S = \Delta S_{solv} + \Delta S_{conf}$$

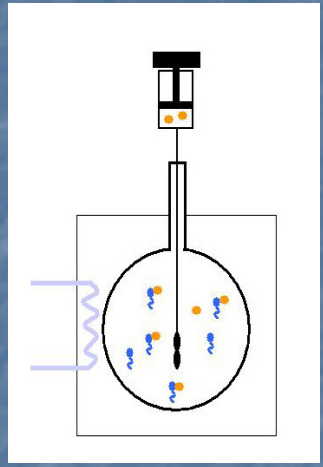
Green: favorable
Red: unfavorable

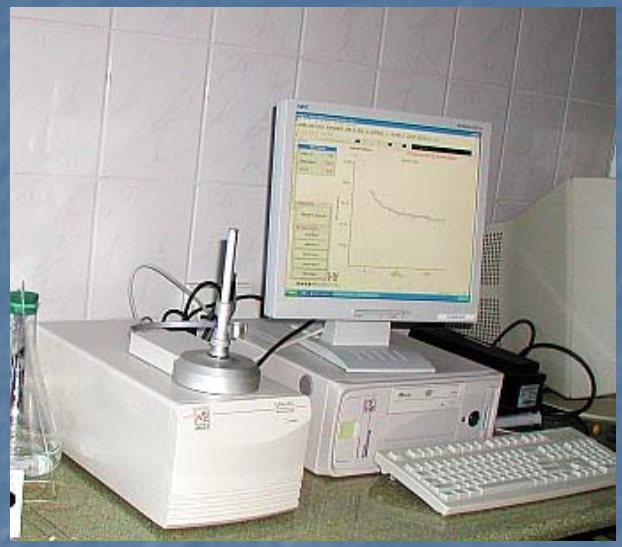
ITC as the gold-standard technique to characterize molecular interactions



Syringe ligand

Cell ligand

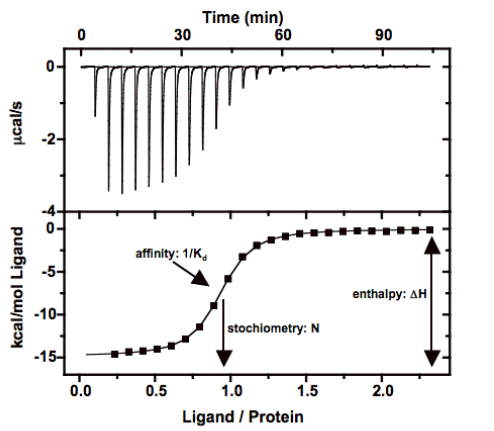




Sample cell ligand needs to be saturated with syringe ligand during experiment

$$\Delta G = - RT \ln K_A = \Delta H - T\Delta S$$

red: measured
blue: calculated
green: constants



Types of interaction studied by ITC…, literally no limits

protein – small molecule
protein – protein
protein – carbohydrate
protein - lipid
protein - DNA
DNA- small molecule
RNA-small molecule
antibody-antigen
DNA-DNA
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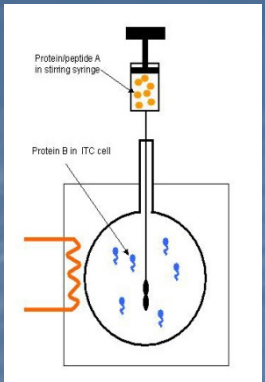
.
.

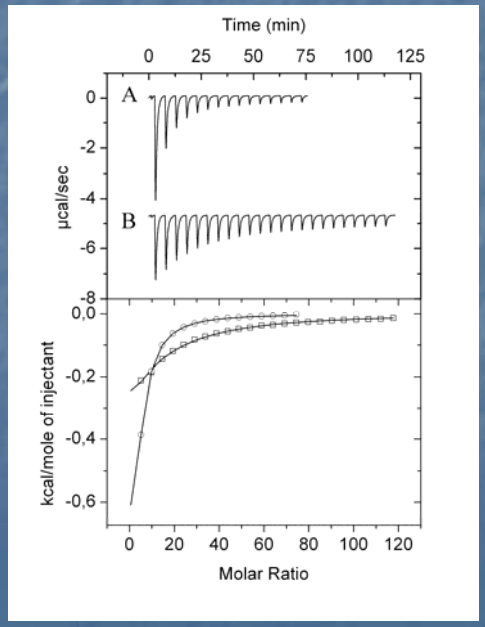
High and low affinity binding events
Simple 1 to 1 binding interactions



[c]:
ligand concentration
in cell

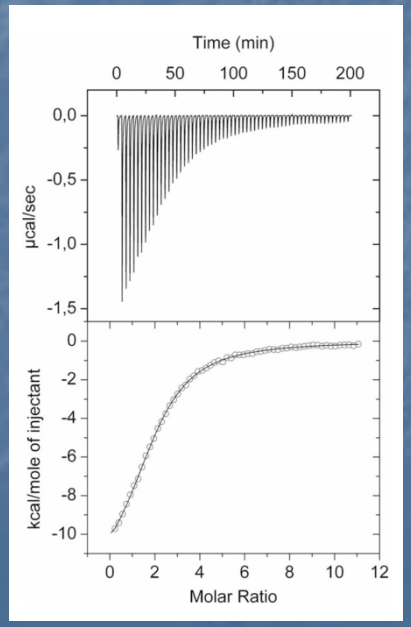
K_D > [c]

Binding of acetate
to McpS-LDB
K_D = 574 µM (A) K_D = 3,3 mM (B)



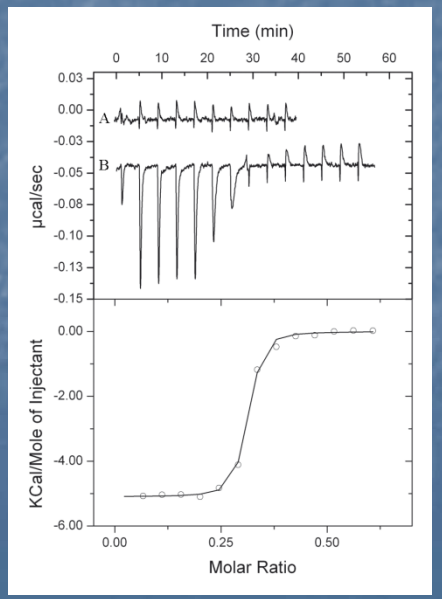
K_D < [c]

Binding of 2-ketogluconate
to PtxS regulator
K_D = 14 µM



K_D « [c]

Binding of phosphate
to phosphate receptor
K_D = 3,7 nM

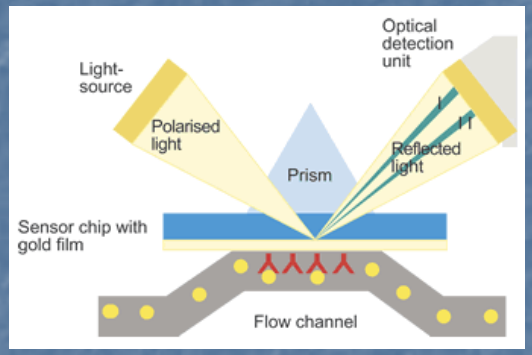


Pineda-Molina et al. (2012) PNAS 109, 18926

Rico-Jiménez et al. (manuscript in preparation)

Advantages/Disadvantage compared to alternative techniques

For example Surface Plasmon Resonance



Advantages

- Both ligands are in solution
- No need for chemical modification
- Heat is a direct consequence of molecular interaction
- Determination of ΔH, ΔS and stoichiometry
- No ligand size restrictions
- Very little restrictions to the buffer
- Analysis temperature between 4 – 80 ºC

Disadvantages

- Relatively large sample amounts needed
- Not possible to analyse complex mixtures (serum)
- No simultaneous determination of different ligands possible

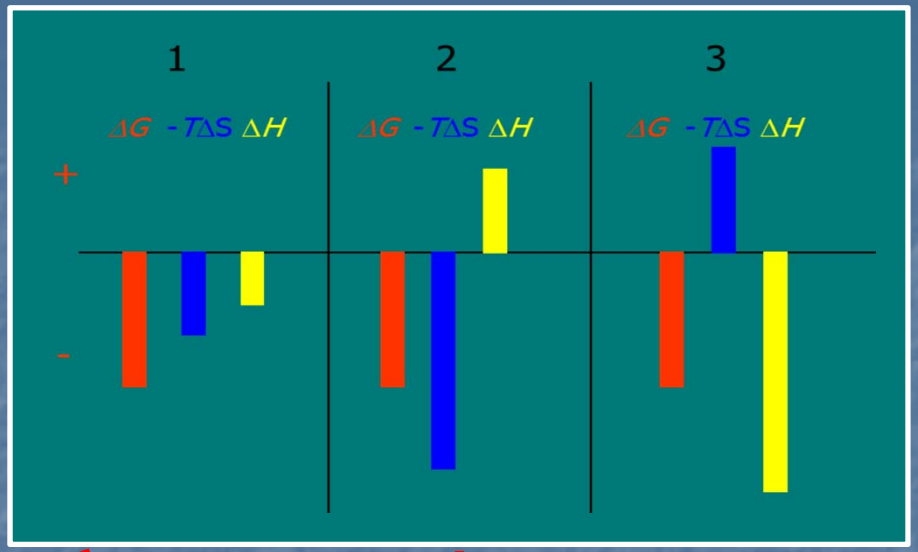
Three different thermodynamic modes of binding

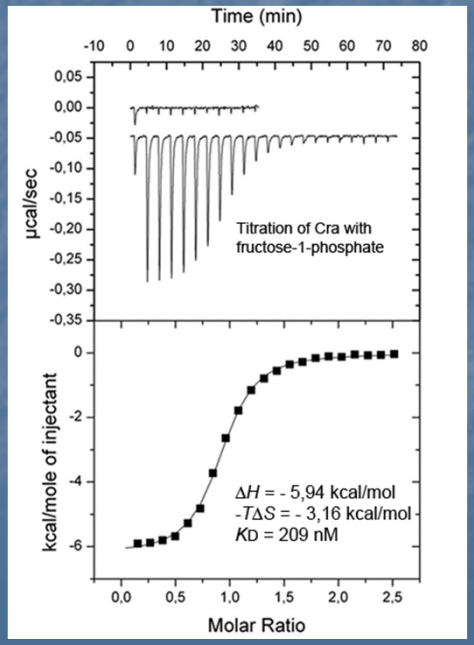
1) Driven by favourable entropy and enthalpy changes

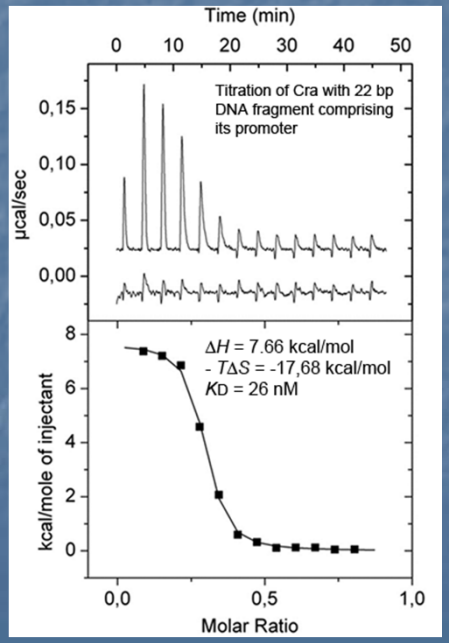
2) Entropy driven.

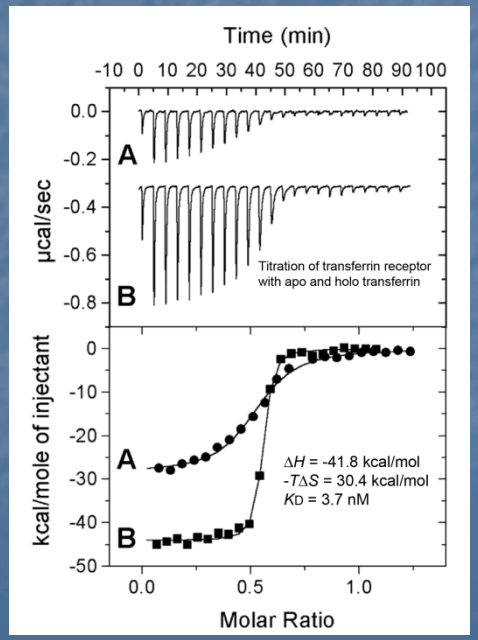
3) Enthalpy driven.

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









Chavarría et al. (2011) J. Biol. Chem. 286, 9351

Krell et al. (2003) J. Biol. Chem. 278, 14712

Use of ITC in the rational optimization of drug lead compounds

Steps in drug development

Target identification
Which proteins/genes
are involved in disease?

Target Validation
Does the inhibition of these
targets reduce disease?

Lead Identification
High throughput screening
with validated target

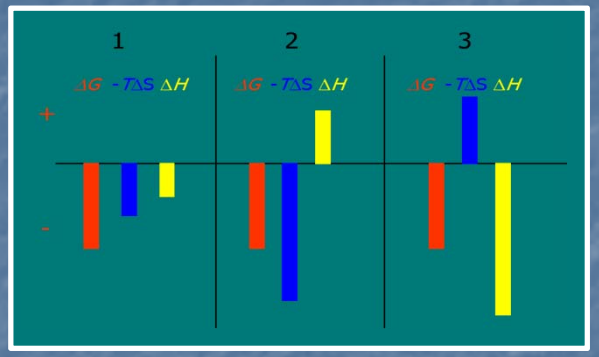
K_D < 10 µM

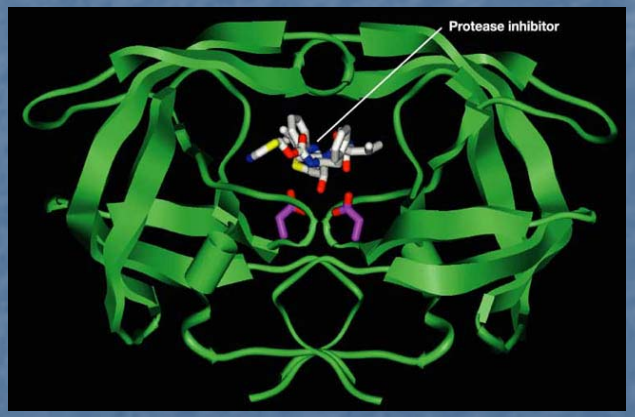
Lead optimization
Increase affinity for target
Increase efficiency

K_D < 10 nM

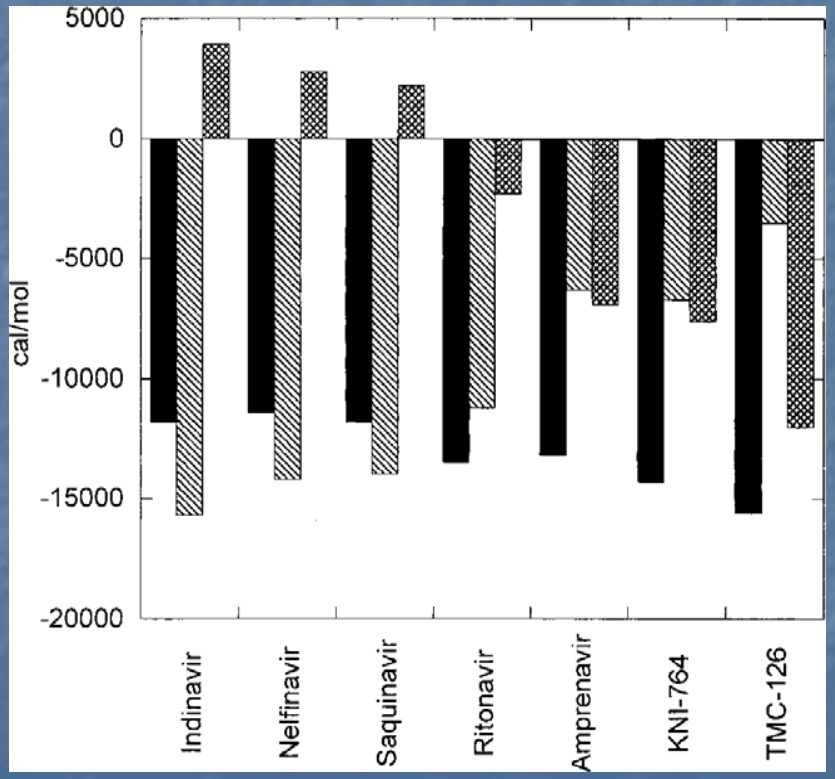
- Typically trial and error approaches
- Major bottleneck

Ultra-tight binding can only be achieved in thermodynamic mode 1





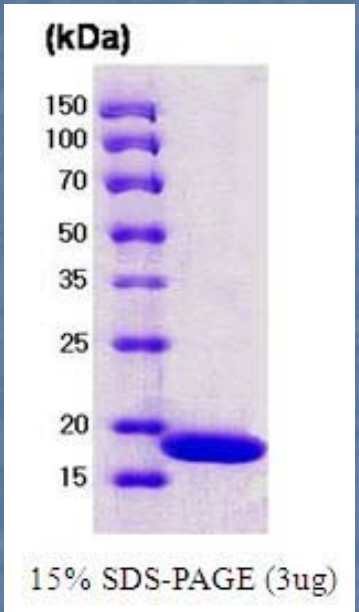
Different inhibitors of HIV proteases

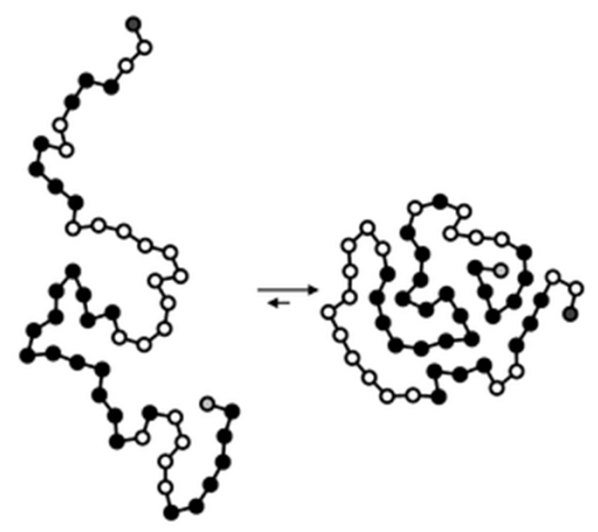


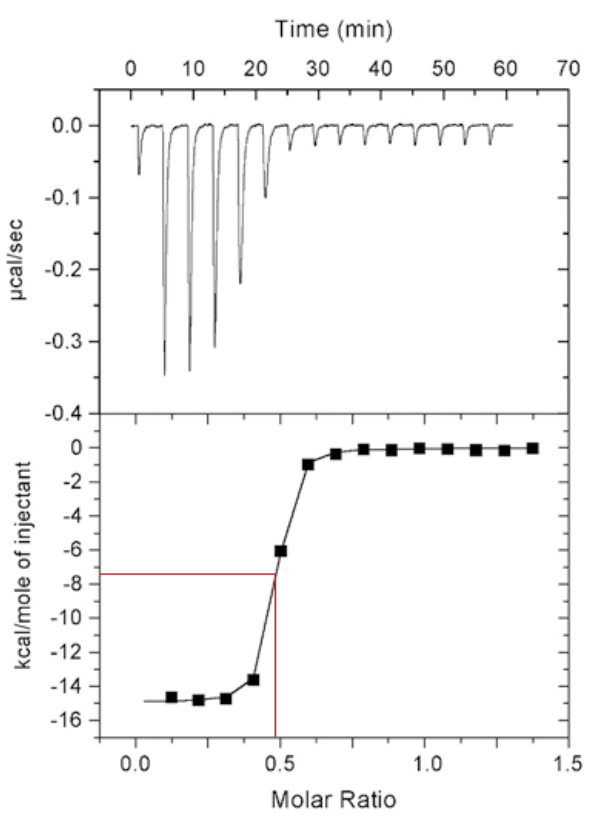
Ohtaka *et al.* (2002) Prot. Sci. 11, 1908.

Exploiting the stoichiometry

Determination of the percentage of active protein



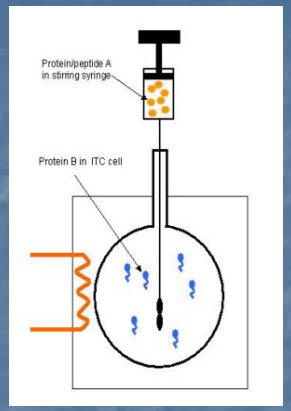




Titration of TbpB with transferrin,
Known to bind with 1 : 1 stoichiometry

Krell et al. (2003) J. Biol. Chem. 278, 14712

Experimental design of an ITC experiment



During en ITC experiment the cell ligand has to be saturated with syrige ligand

Which ligand goes where?

- Solubility
- Sample availability
- Dissociation effects

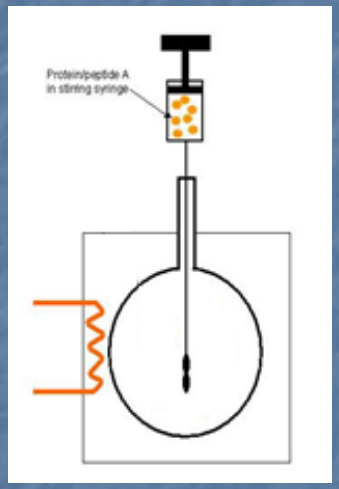
In the absence of any information on interaction:

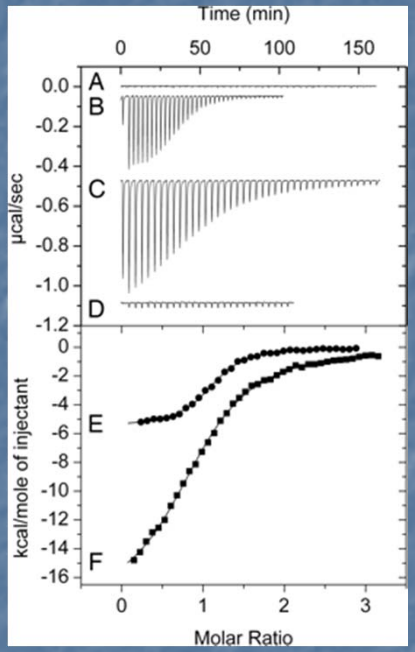
- Initial cell ligand concentration: 10 μM
- Initial syringe ligand concentration: 2 - 3 mM (final concentration in cell: 350 – 500 μM)

Experimental design of an ITC analysis

Essential prerequisite: the injection of syringe ligand into buffer must not produce significant heat changes

= essential control





A: Titration of buffer with 1 mM toluene

B: Titration of TodS with 1 mM toluene

Limit (for us): 0.1 µcal/sec

Non-specific dilution heats can be caused by:

1. Syringe and cell ligand are in different buffer systems
2. The pH of both buffers is different
3. The syringe ligand dissociates
4. Syringe concentration is too high

Preparation of ligands

1. Both ligands are macromolecules
   - dialysis into the same bath of buffer

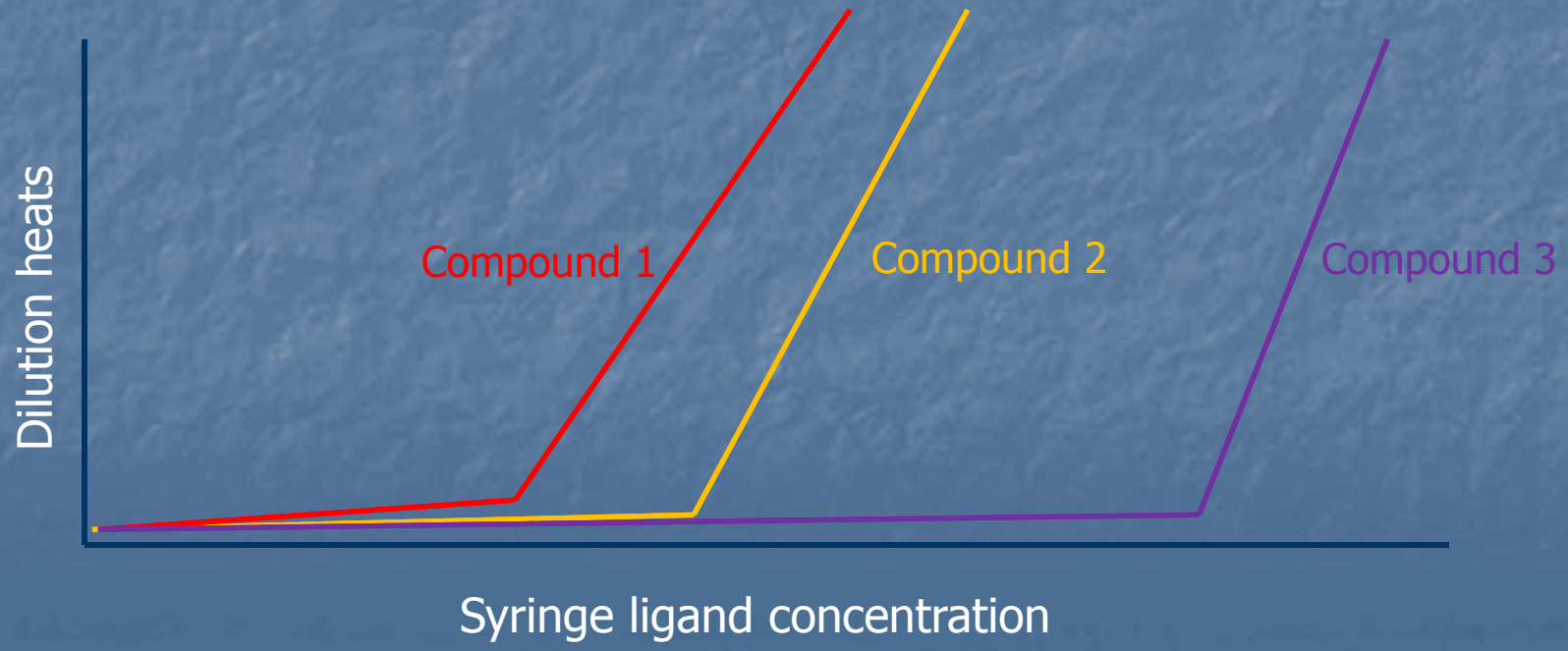
2. One macromolecule, one low molecular weight molecule
   - dialysis of the macromolecule, make up low molecular weight solution with dialysis buffer

3. Both are low molecular weight compounds
   - make up solution in the same buffer

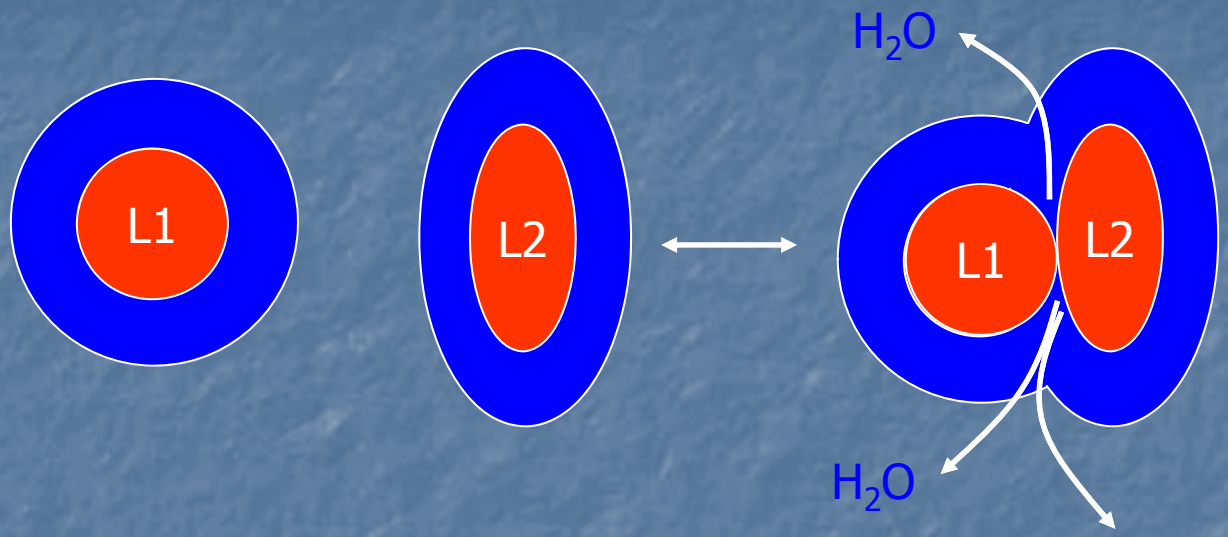
- If compounds are pH active, readjust pH

- If syringe ligands dissociates upon injection (protein multimers), it has to be placed into the sample cell

Dilution heats of syringe ligands

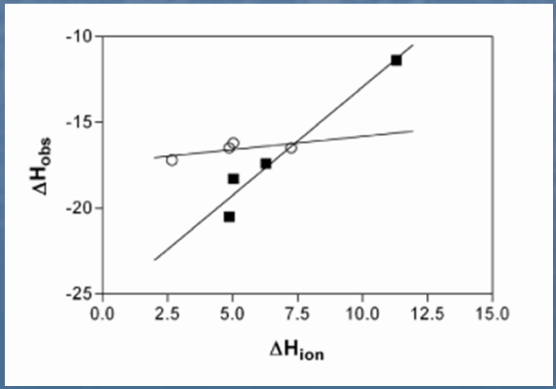


The proton transfer issue: Distorsion of the enthalpy term



$H^+ + buffer = buffer\text{-}H^+$

- Buffers differ in their ionization enthalpy
- To see whether an interaction involves proton transfer, conduct experiments in different buffers



Correction using experiments conducted in 4 different buffers

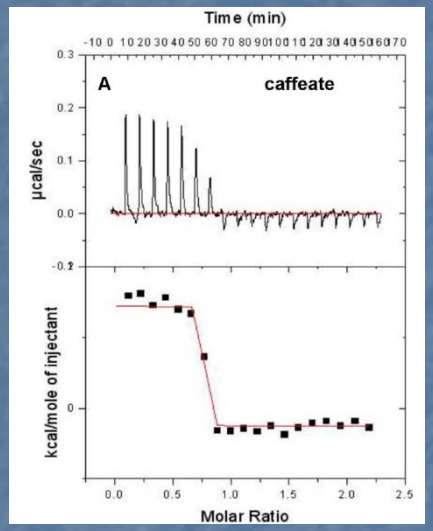
What about little soluble ligands?

For example: hydrophobic compounds, drugs with a very reduced solubility in aqueous buffers

- Preparation of stock solution in 100 % DMSO (i.e. 10 mM ligand in 100 % DMSO)
- Ten-fold dilution using dialysis buffer (final concentration: 1 mM ligand in 10 % DMSO)
- Important: addition of DMSO to dialysed ligand
- Addition of DMSO normally slightly reduces affinity, but permits an analysis

How can I study ultra-tight or very low binding events?

Ultra-tight binding:
only one point at the steep raising part of the curve



No data analysis possible

Salmon et al. (2013)
Plos One 8, e59844

Displacement technique:

- Two ligands with different affinities compete for binding at the same macromolecule

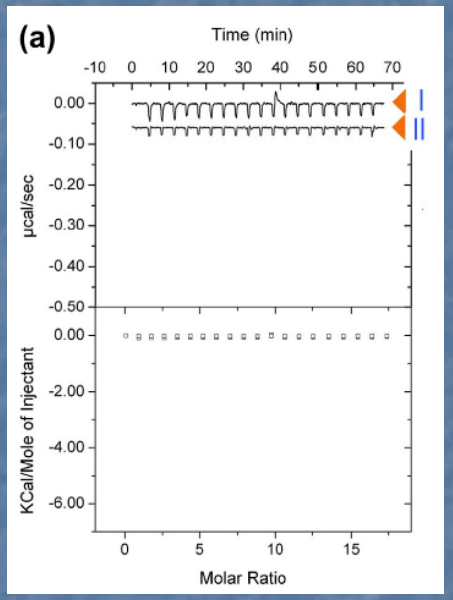
- Initial titration with lower affinity ligand is followed by titration with ultra-tight binding ligand

More information: Sigurskjold (2000) *Anal. Biochem.* **277**, 260–266
Velazquez-Campoy & Freire (2006) Nature Protocols 1:186-91

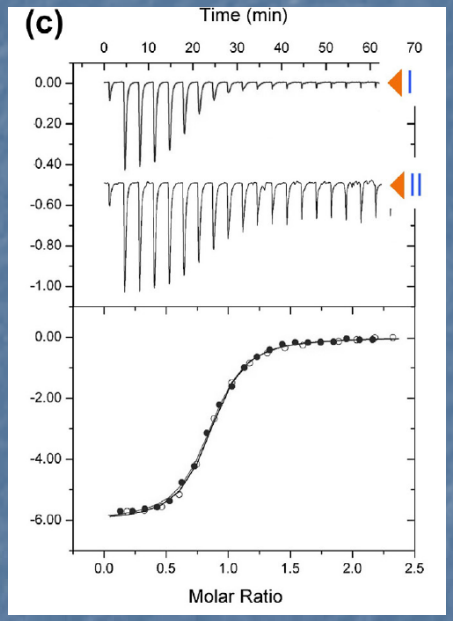
A similar approach can be used to determine whether ligands bind with very low affinity.

Example: Does fructose 1,6-bisphosphate bind with low affinity to Cra?

No binding seen in titration of Cra with 1 mM FBP (maximal concentration)



Titration of Cra with fructose-1-phosphate in the absence (I) and presence (II) of 5 mM fructose 1,6-bisphosphate

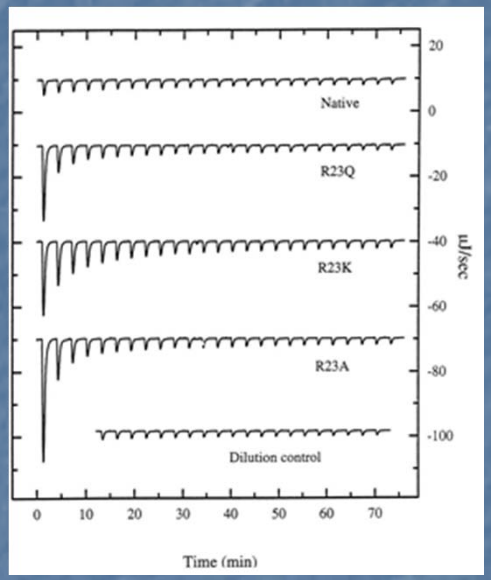


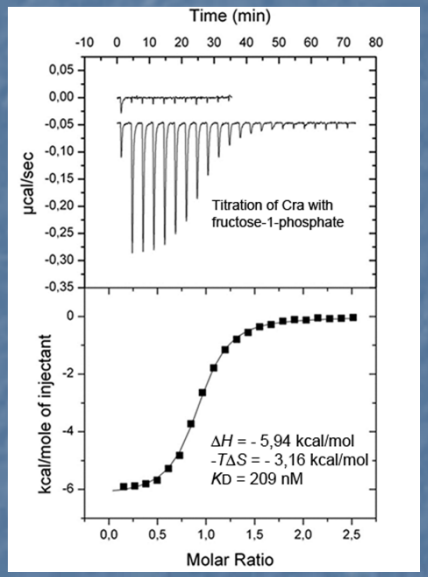
But final cellular FBP concentration was only of 175 µM

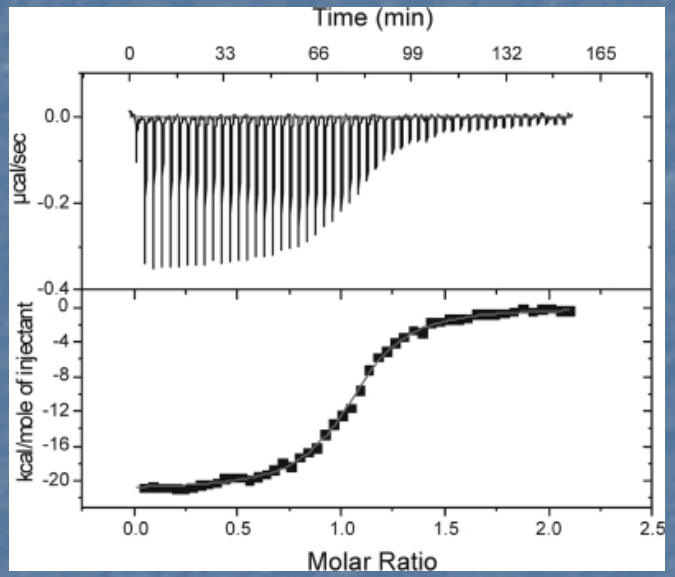
Chavarría et al. (2014) FEBS Open Bio. 4:377-86

Optimisation of an ITC experiment

- The number of peaks is determined by volumes and concentrations
- The ideal trace has approx. 15 data points







Too few points

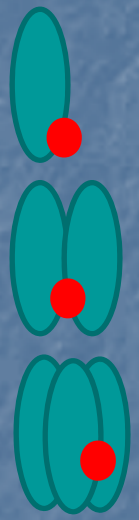
correct

Too many points

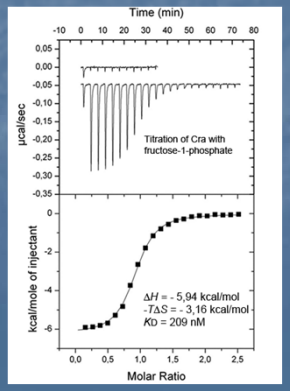
Data analysis

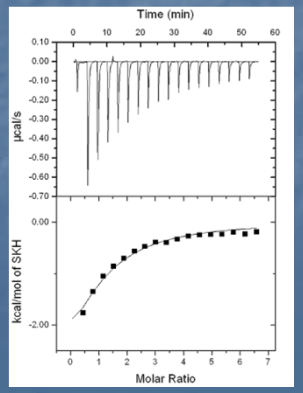
Different mathematical algorithms exist for different binding types

A single type of binding site



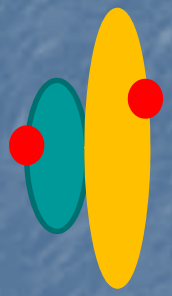
Monophasic curves





Multiple binding sites

Two independent sites



Frequently for hetero-dimers

Two dependent sites

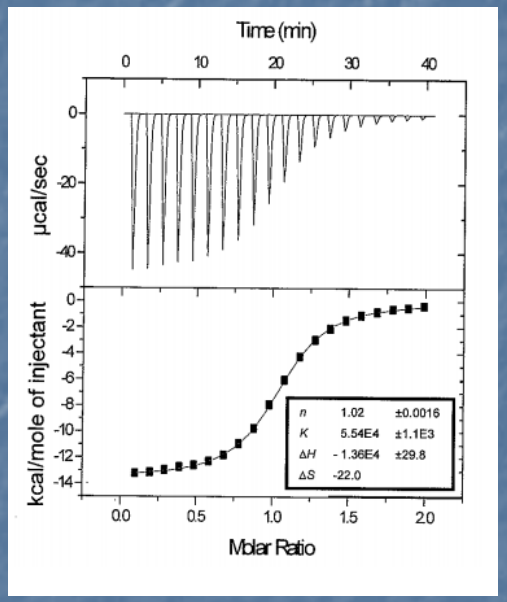
Positive cooperativity

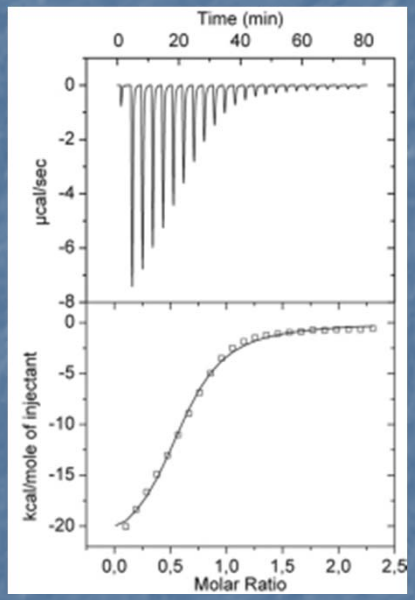
Negative cooperativity

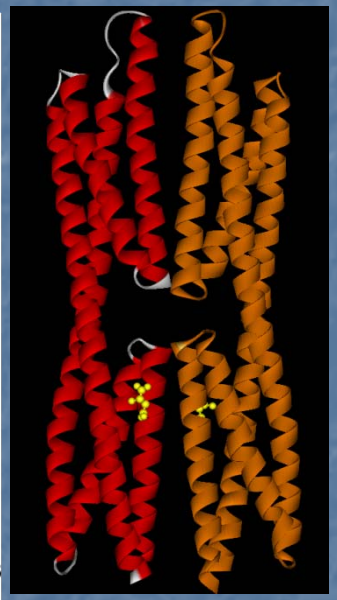
Frequently for protein homodimers or palindromic DNA sequences

Monophasic curves

- All single binding site interactions give rise to monophasic curves
- Not all monophasic curves are single binding site interactions







Titration of ligand binding domain
of McpS chemoreceptor with malate

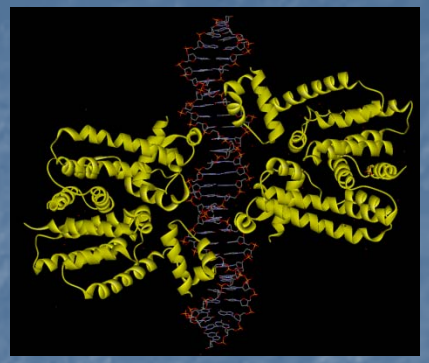
Single binding site

Binding with negative cooperativity

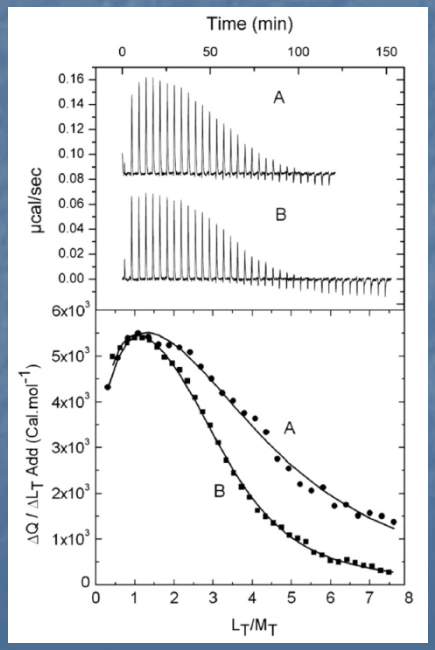
Pineda-Molina et al. (2012) PNAS 109:18926

Multiple binding events

Cooperativity



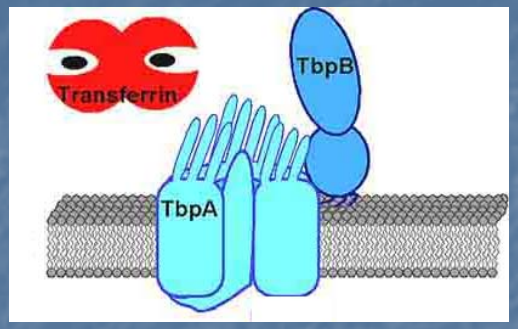
Titration of operator DNA with TtgR

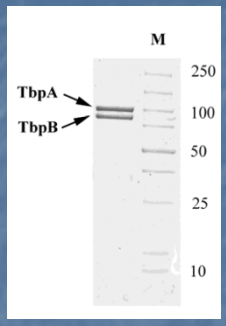


K_{d1} = 18 µM
K_{d2} = 0,9 µM

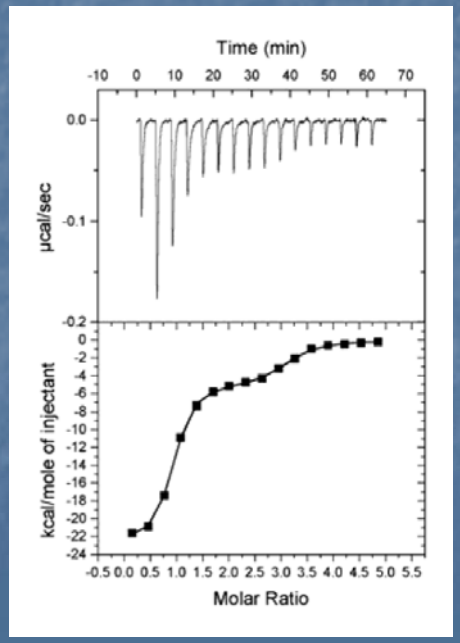
Krell et al. (2007) J Mol Biol. 369, 1188

Independent sites





Titration of transferrin receptor (TbpA & TbpB) with transferrin



K_{D1} = 0.7 nM
K_{D2} = 22 nM

Krell et al. (2003) J. Biol. Chem. 278, 14712

Distinguishing good from bad ITC data

Titration of ArcA with TolC

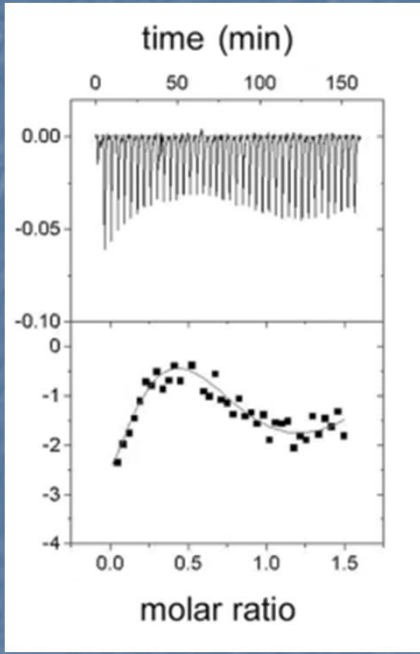


Table 1. Thermodynamic parameters of interactions.

Proteins	Phases	K_a	ΔH	ΔS	ΔG
TolC/AcrA	4	$5.2 \pm 1 \times 10^5$	-2.9 ± 0.2	16	-7.8 ± 1
		$7.6 \pm 3 \times 10^4$	3.9 ± 1.0	153	-6.6 ± 2
		$3.2 \pm 1 \times 10^5$	-99 ± 20	-310	-6.6 ± 2
		$5.8 \pm 3 \times 10^4$	192 ± 50	670	-6.4 ± 3

- Data analysis using a model with 4 independent events (model with 12 variables, 4 x *K*, n and ΔH)

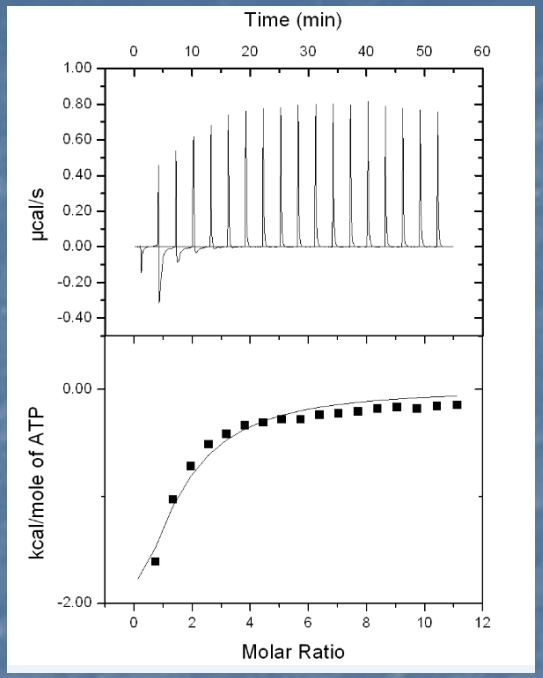
- Most negative enthalpy change ever measured for protein-protein interaction: -65 kcal/mol

- Most positive enthalpy change recorded: 35 kcal/mol

Mol Microbiol. (2004) 53:697-706.

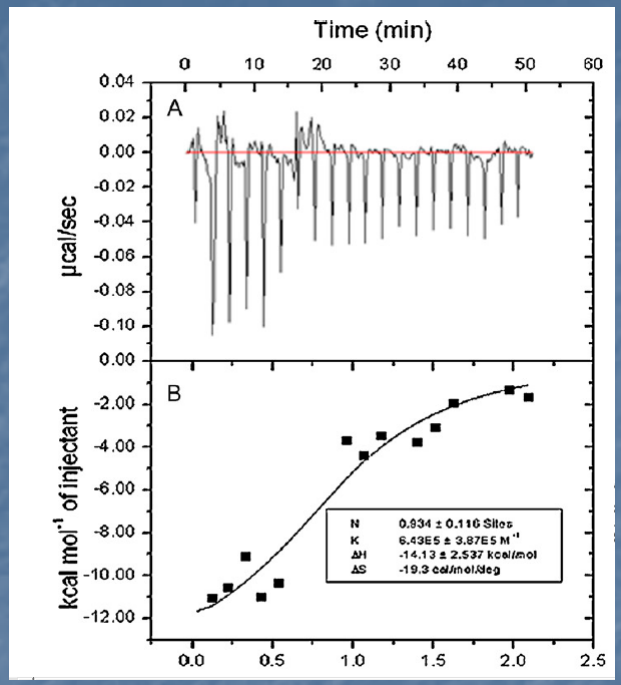
- Very weak signals
- No control
- Do analyse data

Distinguishing good from bad ITC data II



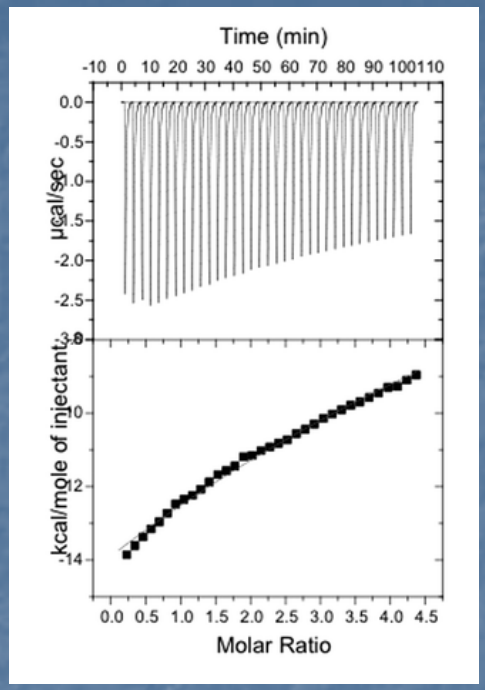
PLoS One. (2013) 8(5):e61918.

- Large dilution heats
- Few points
- Bad dilution heat correction



Biochem. Pharmacol. (2013) 84, 633

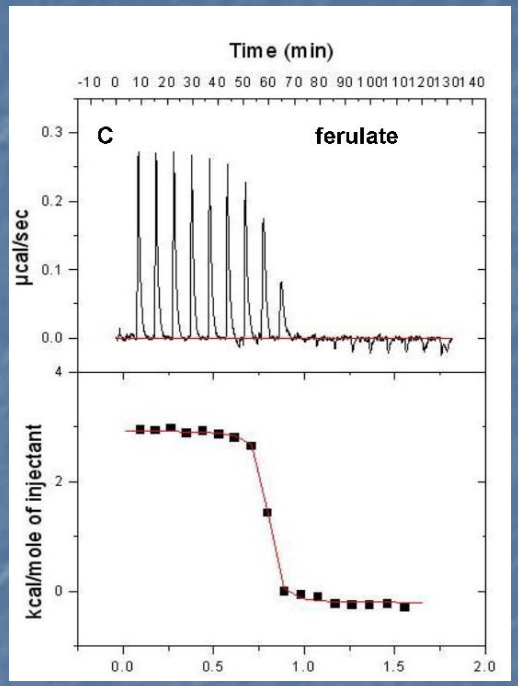
- Noisy data
- Bad peak integration
- No correction for dilution heats

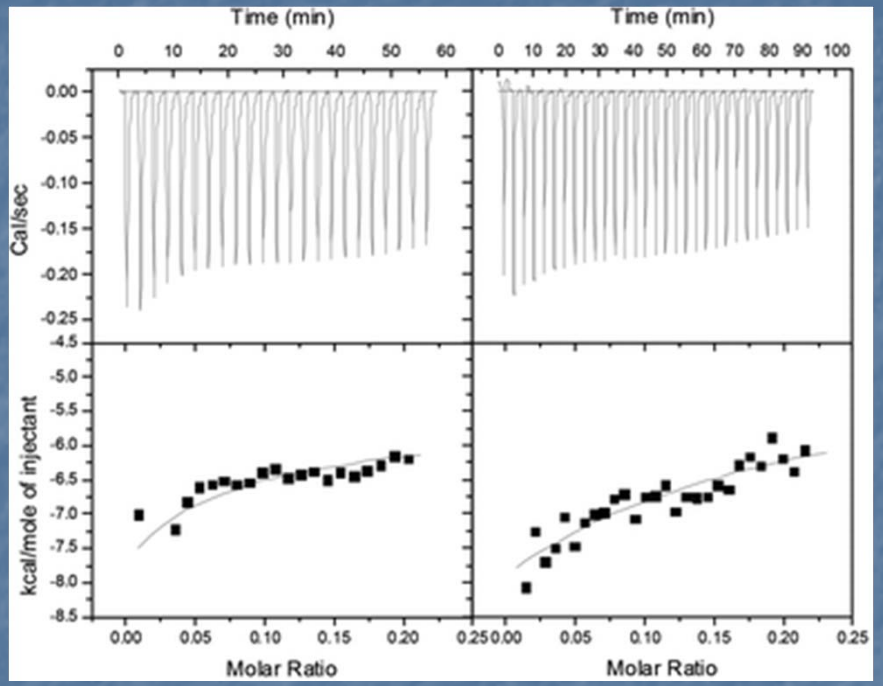


Chem. Biol. (2004) 11, 1127–1137

- No saturation

Distinguishing good from bad ITC data III





PLoS One. (2013) 8:e59844

- Calculation of parameters with only one point at steep rising part of the curve

Dalton Trans. (2014) **43**, 9216-9225

- No controls
- No saturation
- No correction

Other uses of titration calorimetry

The Thermomix of the biochemist

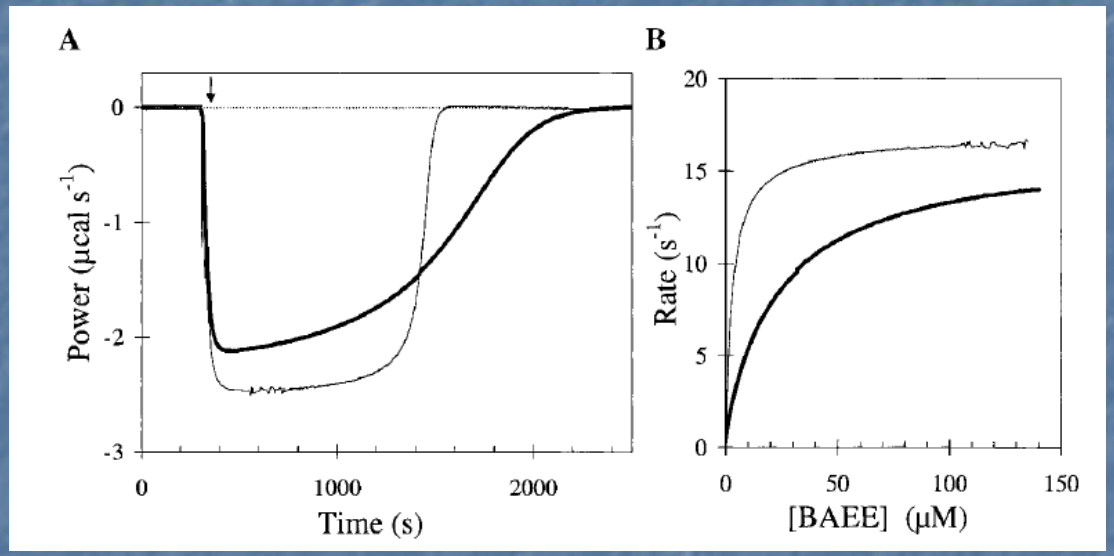
- Universal enzyme assay
- To measure dissociation
- To follow chemical reactions

ITC as an universal enzyme assay

Analytical Biochemistry **296**, 179–187 (2001)
doi:10.1006/abio.2001.5218, available online at http://www.idealibrary.com on IDEAL®

Enzyme Kinetics Determined Using Calorimetry: A General Assay for Enzyme Activity?[1]

Matthew J. Todd[2] and Javier Gomez[3]
Department of Biology and Department of Biophysics, The Johns Hopkins University, Baltimore, Maryland 21218



Single injection of substrate into trypsin in the absence (thin line) and presence (thick line) of protease inhibitor.

Conversion into Michaelis Menten plot

Kinetic constants determined by ITC are similar to those determined by standard assays

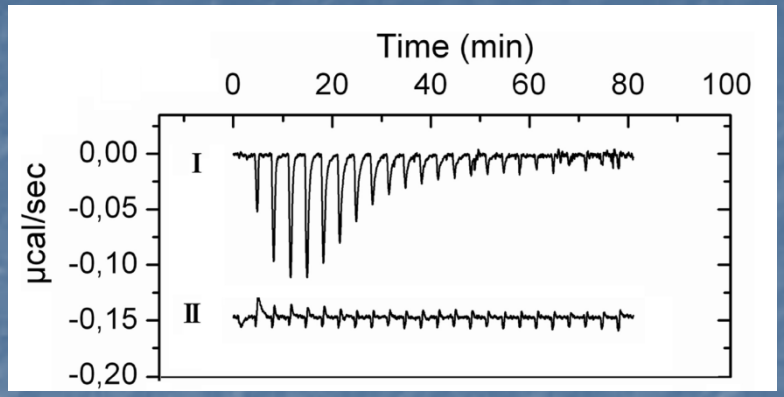
TABLE 1

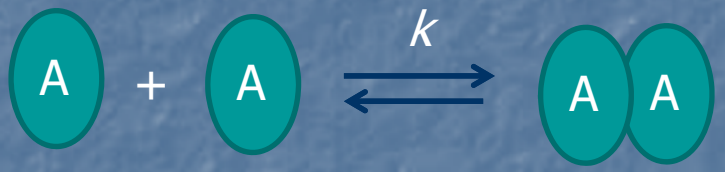
Kinetics of Enzymes Assayed Calorimetrically versus Published Values

Enzyme	Calorimetric		Literature values	
	K_m	k_{cat}	K_m	k_{cat}
EC 1.5.1.3 (DHFR)[a]				
Substrate = DHF	1.2 μM	6 s^{-1}	6 μM	3 s^{-1} (35)
EC 2.7.1.1 (yeast hexokinase)[b]				
Substrate = glucose	72 μM	270 s^{-1}	100 μM	450 s^{-1} (36)
EC 3.3.2.6 *B. cereus* penicillinase I[c]	120 μM	3600 s^{-1}	50 μM	2800 s^{-1} (37)
EC 3.4.21.4 (trypsin)[d]	4 μM	15 s^{-1}	5 μM	22 s^{-1} (38)
EC 3.4.21.16 (HIV protease)[e]	5–300 μM [NaCl] dependent	10 s^{-1}	15 μM	45 s^{-1} (33)
Substrate = KARVnLF(NO_2)EAnL				
Substrate = VSQNYPIVQ				
EC 3.5.1.5 (*H. pylori* urease)[f]	0.79 mM	1400 s^{-1}	0.17 mM	2700 s^{-1} (39)
EC 4.1.1.7 (*F. heparinum* heparinase)[g]	1.8 μM	0.059 s^{-1}	10.2 μM	92 s^{-1} (40)
EC 4.1.1.39 (rubisco)[h]				
Substrate = ribulose bisphosphate	0.15 mM	1.95 s^{-1}	0.053 mM	1.76 s^{-1} (41)
EC 4.1.3.18 (acetolactate synthase)[i]	4.8 mM	11 s^{-1}	5.5 mM	5.3 s^{-1} (42)
EC 5.99 (GroEL)[j]	3 μM n = 2.9	0.052 s^{-1}	5 μM n = 2.5	0.08 s^{-1} (22)
EC 6.4.1.1 (pyruvate carboxylase)[k]				
Substrate = ATP	85 μM		58 μM	
Substrate = pyruvate	105 μM		440 μM	(43)

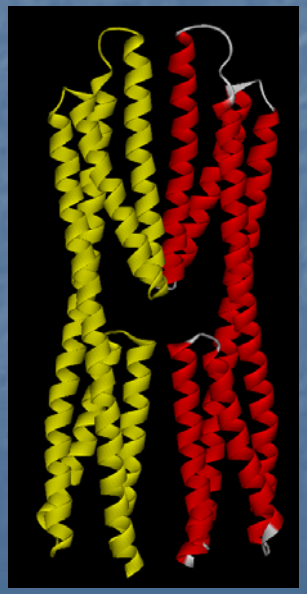
Todd & Gomez (2001) Anal. Biochem. 296, 179.

ITC to measure dissociation/self association



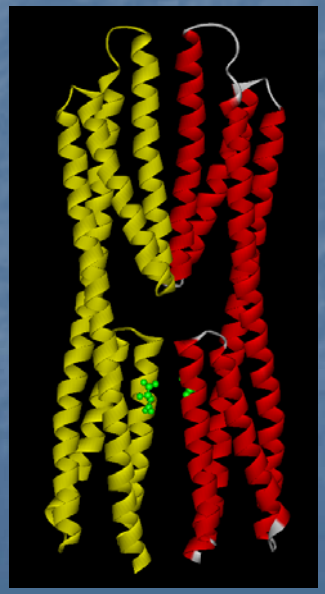


I

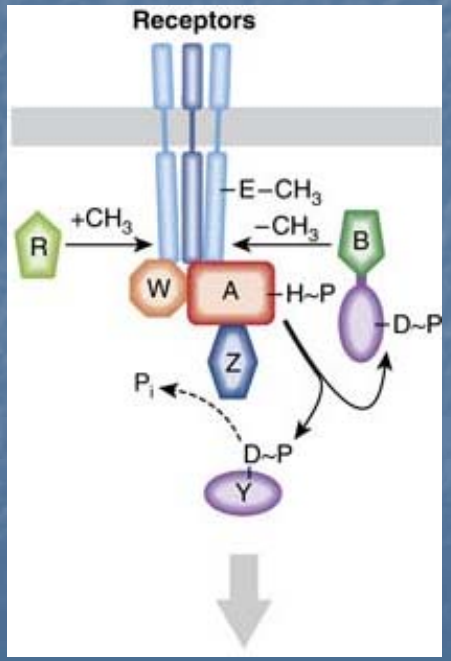


Loosly associated dimer

II

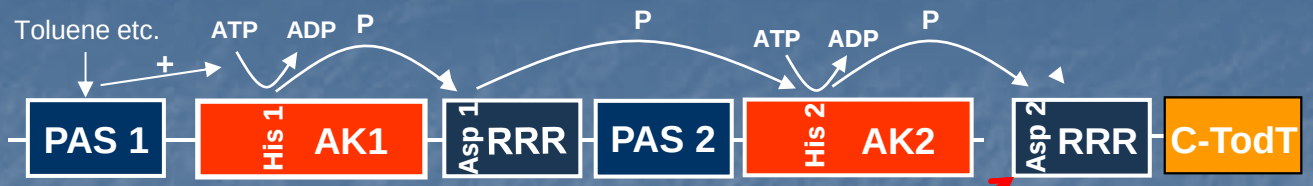


Tightly associated dimer

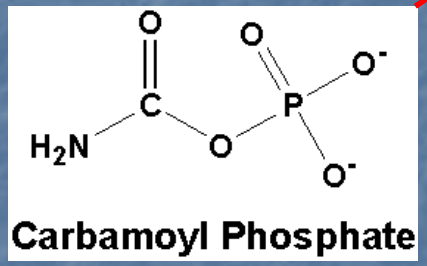


Pineda-Molina et al. (2012) PNAS 109:18926

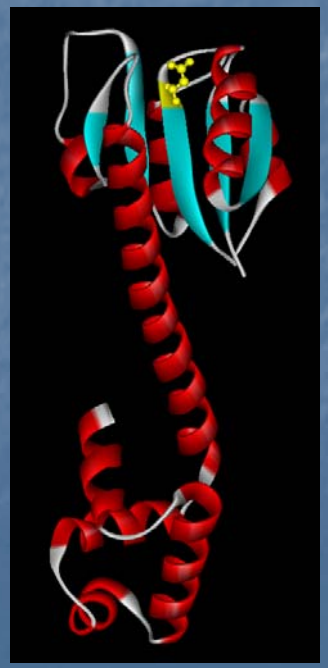
ITC to follow chemical reactions

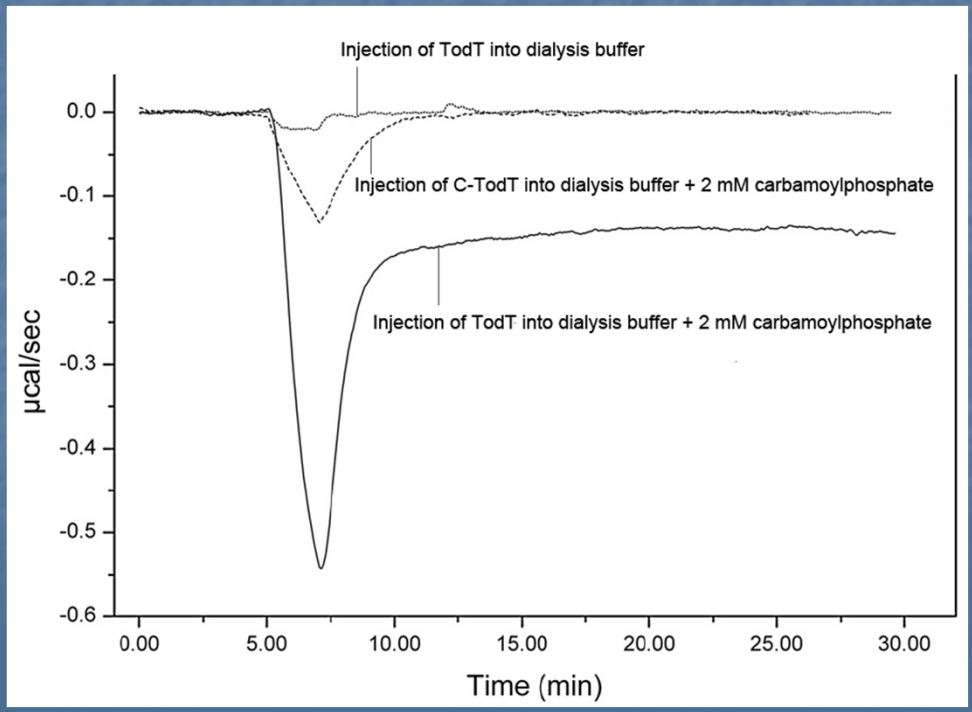


?



Carbamoyl Phosphate





Lacal et al. (2008) J. Mol. Biol. 376, 325

ITC: tendencies

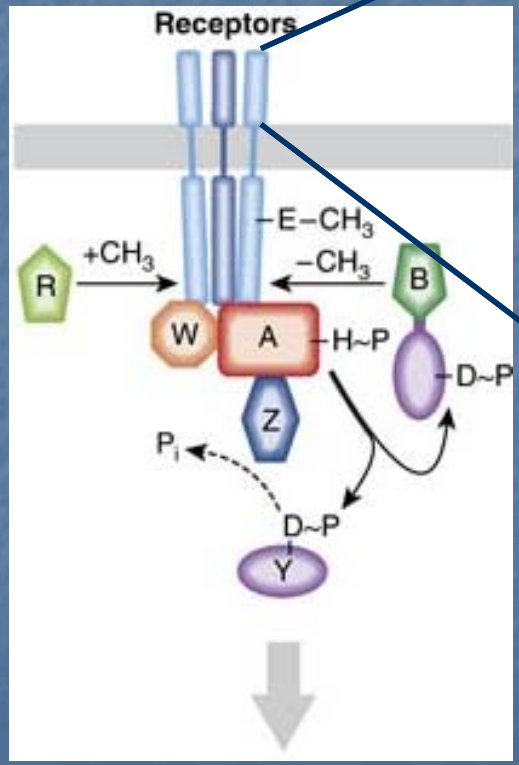
Instrument manufacturers address the weak points of this technique (particularly for companies):

- Labour intensive
- High sample amounts necessary

Development of an ITC robot: Auto ITC

- Higher sensitivity, smaller cells, lower sample amount necessary
- Automated conduct of experiments
- Automated analysis

Titration of the ligand binding domain of the PctB chemoreceptor with L-Gln



Rico-Jiménez et al. (2013) Mol. Microbiol. 88:1230