Applications of Microcalorimetry in Biomolecular Interactions

Prof. Dr. Peter Macheroux
Institut für Biochemie
Email: peter.macheroux@tugraz.at
http://www.biochemistry.tugraz.at

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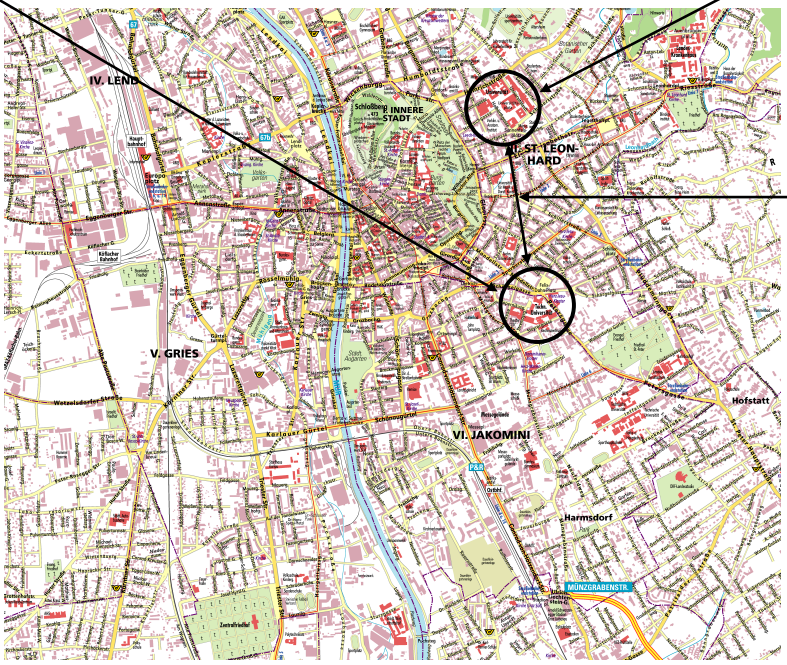


Ca. 800 m

Cultural city of Europe in 2003

Molecular Interactions of Biomolecules

1) Small molecule (< 1000 Da) to large molecule

Examples: substrate to active site of enzyme; effector to allosteric site; inhibitor to active site; prosthetic group to enzyme; signaling molecule to receptor

2) Large molecule to large molecule

Examples: protein-protein interaction; antigen-antibody binding; major histocompatibility complex (MHC) to T-cell receptor; DNA/RNA to proteins

Physical Properties of Binding

Thermodynamic properties:

Equilibrium concentrations of reactants and products are governed by:

$\Delta G = \Delta H - T\Delta S$, with ΔH = enthalpy, ΔS entropy

Also informative:
heat capacity (Cp) – temperature dependence of ΔH

Thermodynamics of Binding

- Binding is favoured, if it leads to a net increase in disorder or entropy, then ΔG becomes negative because of the $T\Delta S$ term (entropically favoured binding)
- Equally, ΔG becomes negative when the binding interaction releases heat ($-\Delta H$, exothermic binding)
- In practice, the enthalpy and entropy term contribute to binding of molecules (note that both terms are temperature dependent and therefore their relative contributions are affected by temperature!)

Kinetic Description of Binding

Kinetic properties:

$$[P] + [L] \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} [PL]$$

the association rate k_{on} (M^{-1} sec^{-1}) and the dissociation rate k_{off} (sec^{-1}) describe the binding interaction (***microscopic rate constants***)

k_{on} and k_{off} define the equilibrium constant (see next slides):

$$K_{eq} = \frac{k_{on}}{k_{off}} \quad [M^{-1}]$$

Affinity

Affinity is a measure of how favourable an interaction is

For the following interaction

$$[P] + [L] \rightleftharpoons [PL]$$

the affinity constant K_A is expressed as:

$$K_A = \frac{[PL]}{[P]\ [L]}$$

- is the ratio of product(s) versus reactants at equilibrium
- unit is M^{-1}
- higher affinity = higher K_A

Dissociation

Most biochemical equilibria are considered in terms of dissociation:

$$K_A = \frac{1}{K_D}$$ K_D = dissociation constant

The dissociation constant can be thought as the ***concentration of P at which half of L is bound ([L] = [PL]):***

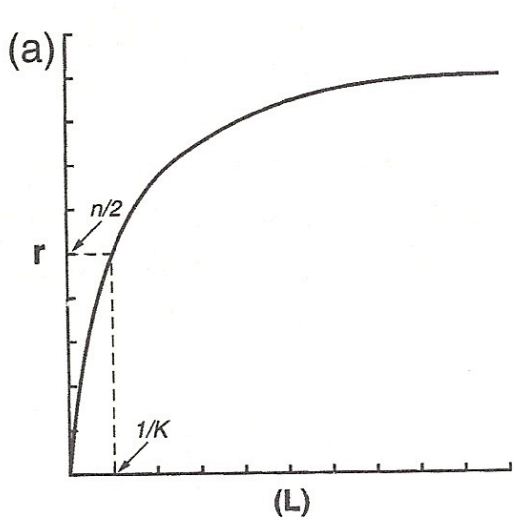
$$K_D = \frac{[P]\ [L]}{[PL]} = \frac{[P]\ \cancel{[L]}}{\cancel{[PL]}} = [P]$$

- unit is M
- higher affinity = lower K_D

note that $\Delta G = RT \ln K_D$

The Binding Isotherm

Binding equilibria are characterized by a binding isotherm where the moles of ligand bound per mole of protein is expressed as r:



$$r = \frac{[PL]}{[P] + [PL]}$$

with $K_A = [PL] / [P]\,[L]$

$$\downarrow$$

$$r = \frac{K_A\,[L]}{1 + K_A\,[L]}$$

a plot of r against [L] is a hyperbolic curve with a limiting value of 1 and at r = 0.5 [L] = $1/K_A$

More Than One Binding Site

If there are **n** equivalent binding sites on the protein, the binding isotherm is the sum of those for each binding site:

$$r = \frac{\mathbf{n}\,K_A\,[L]}{1 + K_A\,[L]}$$

A plot of r against [L] has the same shape with a limiting value of **n** and when r = **n**/2 than [L] = $1/K_A$

Thus a study of ligand binding to a protein reveals the number of binding sites and the affinity of the ligand

To facilitate analysis of binding data the equation describing ligand binding can be linearized in several ways (see next slides). In the age of desktop computers this is not really necessary because hyperbolic data can be fitted using non-linear least square programs

How to Measure Binding

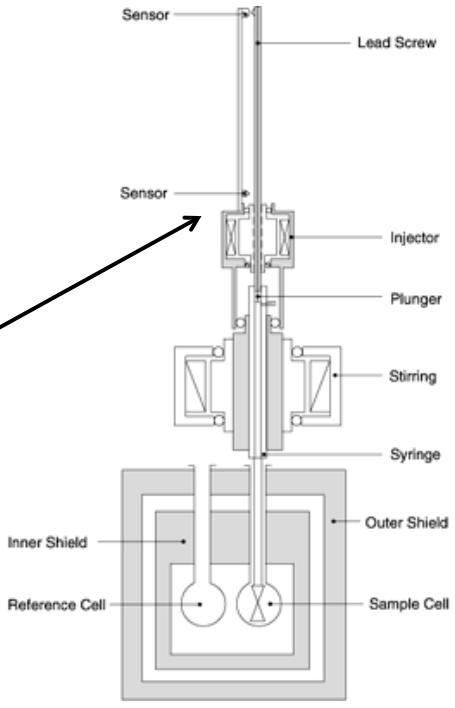
- **Direct measurement** of the concentration of free and bound ligand as well as the concentration of the complex at equilibrium
- Measurement of the **change of a physical or biological property** of the ligand or the protein when binding occurs
- Kinetic measurement of the rate constants for k_{on} and k_{off}

Microcalorimetry

Isothermal titration calorimetry (ITC)

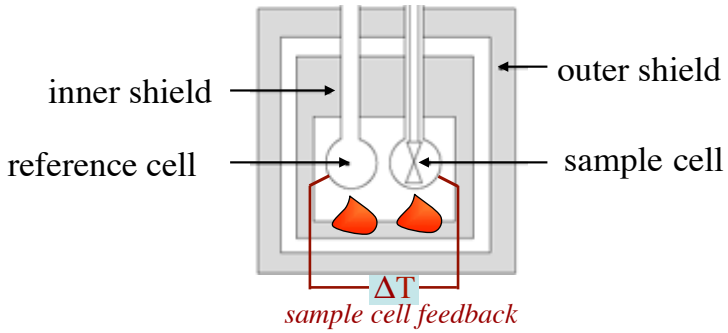
- sensitive technique for measuring heat evolved or absorbed in a sample
- prerequisite is a measurable enthalpy associated with the binding process

spinning syringe

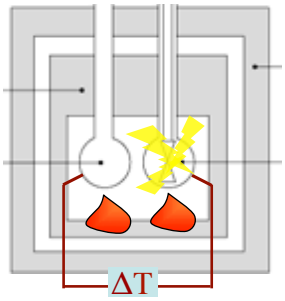


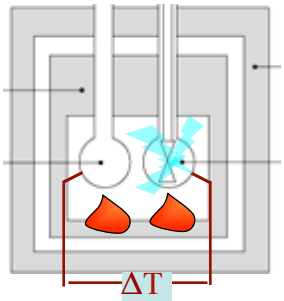
Sample Cell Feedback

- two identical cells
- reference cell is filled with water
- adiabatic jacket, T lower than operating temperature
- both cells are constantly heated to working temperature



Exothermic & Endothermic Reaction





Setting up of an ITC Experiment

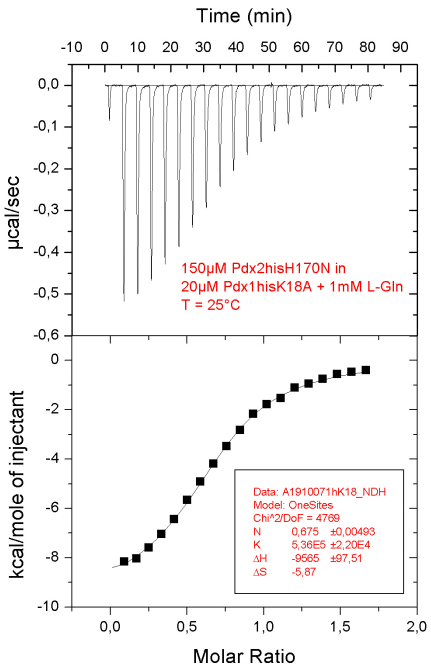
1. titration
   - sample cell: macromolecule solution
     at least 3 μM of macromolecule for tight binding
   - syringe: ligand solution
     concentration of ligand at least 30 μM

➔ Both binding partners (*e.g.* macromolecule and ligand) have to be dissolved in ***exactly*** the same buffer without any interfering components.

2. reference titration (*e.g.* add ligand to buffer)

3. data interpretation

Raw Data & Interpretation



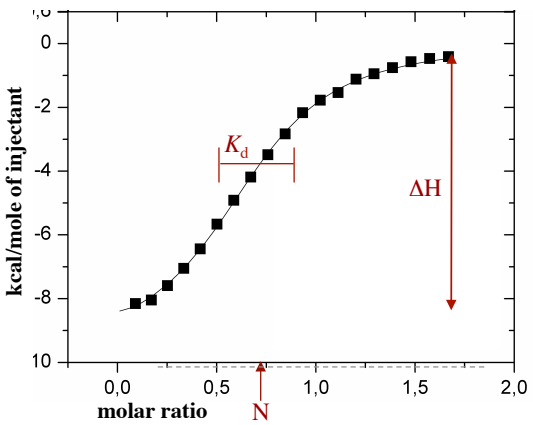
- raw data: heat of injections
- baseline adjustment
- integration yields binding isotherm
- reference subtraction for considering heats of dilution, etc.
- data fitting to obtain
  - **N, K_d, ΔH**, ΔS, ΔG

Thermodynamic Data Interpretation

$$K_d = \frac{1}{K_a}$$

$$\Delta G = -RT \cdot \ln K_a = RT \cdot \ln K_d$$

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



ΔG: Gibbs Free Energy

ΔG < 0 for spontaneous processes

ΔH: Enthalpy

heat consumed or evolved by chemical reactions

ΔH < 0 exothermic

ΔH > 0 endothermic

ΔS: Entropy

measure for the degree of order in a system mainly influenced by the displacement of H_2O

ΔS > 0 desolvation

ΔS < 0 structural confinement

K_d: Dissociation constant

strength of interaction

Advantages & Disadvantages of ITC

Advantages:

- label-free, no immobilization required
- native proteins in „natural environment“ (buffer solution)
- full thermodynamic profile in a single experiment
- no molecular weight limitations

Disadvantages/Restrictions:

- (high) solubility of binding partners
- binding partners need to be soluble in same buffer system
- requirement of large quantities of proteins
- K_d in the nM to mM range
- change in ΔH is a prerequisite

Examples for ITC measurements

- Dipeptidylpeptidase III
- Alkylsulfatase, PISA1
- Putative protease from *Bacteroides thetaiotaomicron*, ppBat
- Quinone reductase, NQO1
- Pyridoxalphosphate synthase complex, Pdx1/2

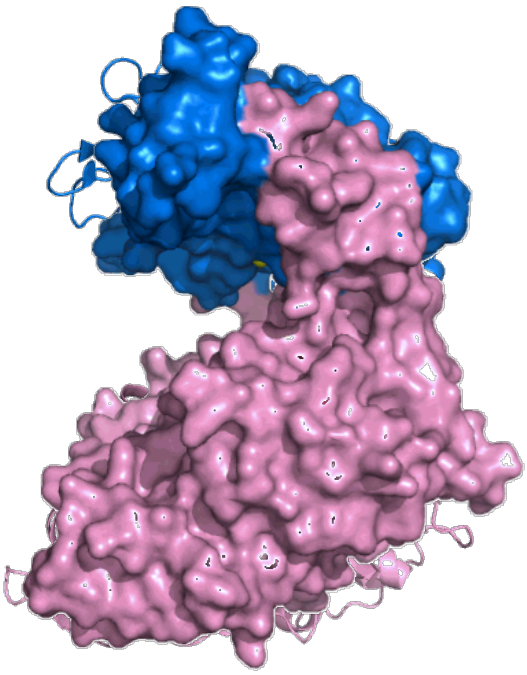
Dipeptidyl-peptidases (DPPs)

- DPPs cleave dipeptides from the amino-terminus of various peptides
- degradation of peptide hormones
- structurally and mechanistically diverse enzyme family
- DPP IV best studied, cleaves glucagon-like peptide 1 (reduces blood glucose levels)
- DPP IV inhibitors (“gliptins”) are hypoglycemics used for the treatment of type 2 diabetes

DPP III

- Zn-dependent amino-dipeptidase, molecular mass: 80-85 kDa
- cleaves dipeptides from the N-terminus of its substrates (optimal length: 4-8 amino acids)
- ***in vitro*:** broad specificity, high affinity for angiotensins, enkephalins and endomorphins
- ***in vivo*:** high concentrations were found in the superficial laminae of rat spinal cord dorsal horn, co-localizes with opioid peptides → potential role in pain modulation
- Part of the **central human proteome** comprising ca. 1000 ubiquitously and abundantly expressed proteins

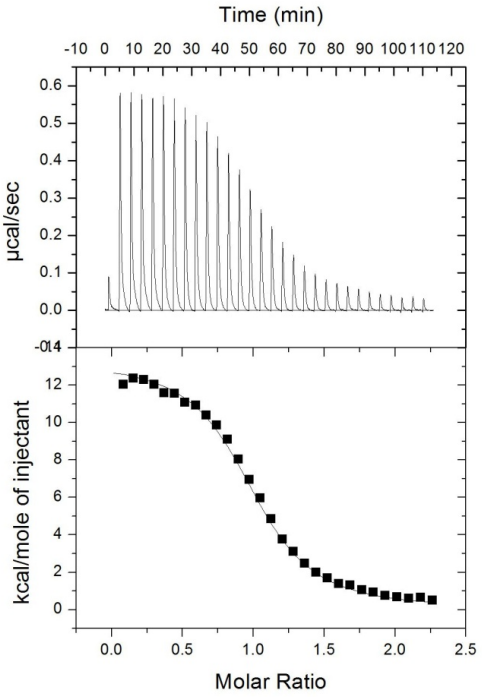
Overall Structure of DPPIII from Yeast



- 1.95 Å resolution
- novel ("orphan") fold
- two domains
- zinc ion bound to the α-helical domain
- peptide binding cleft between the two domains

Baral *et al.*, *J. Biol. Chem.* **283**:22316-22324, 2008

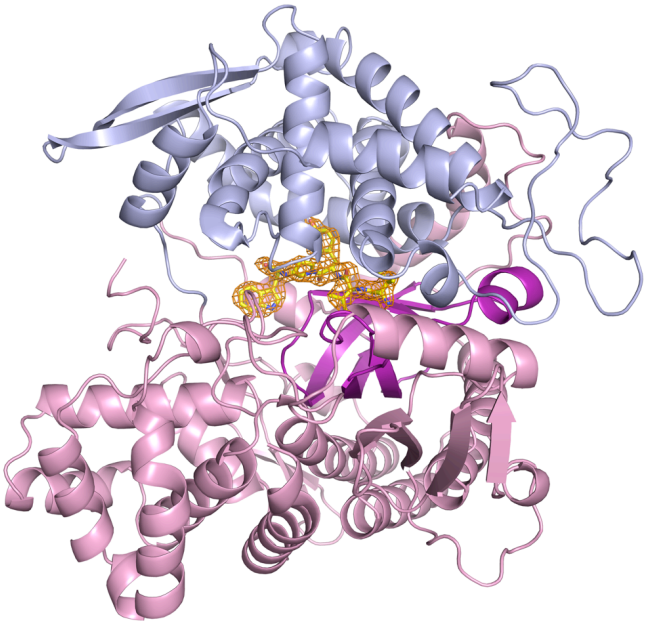
Thermodynamics of Peptide Binding

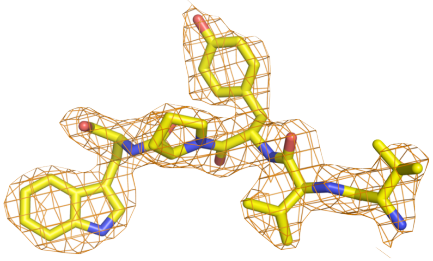


- Isothermal titration calorimetry measurements for the binding of VVYPW (tynorphin)
- Endothermic process
- K_D=1.2 µM
- Entropy gain as the driving force

Bezerra *et al.*, *PNAS* **109**:6525-6530, 2012

Human DPP III* in Complex with VVYPW

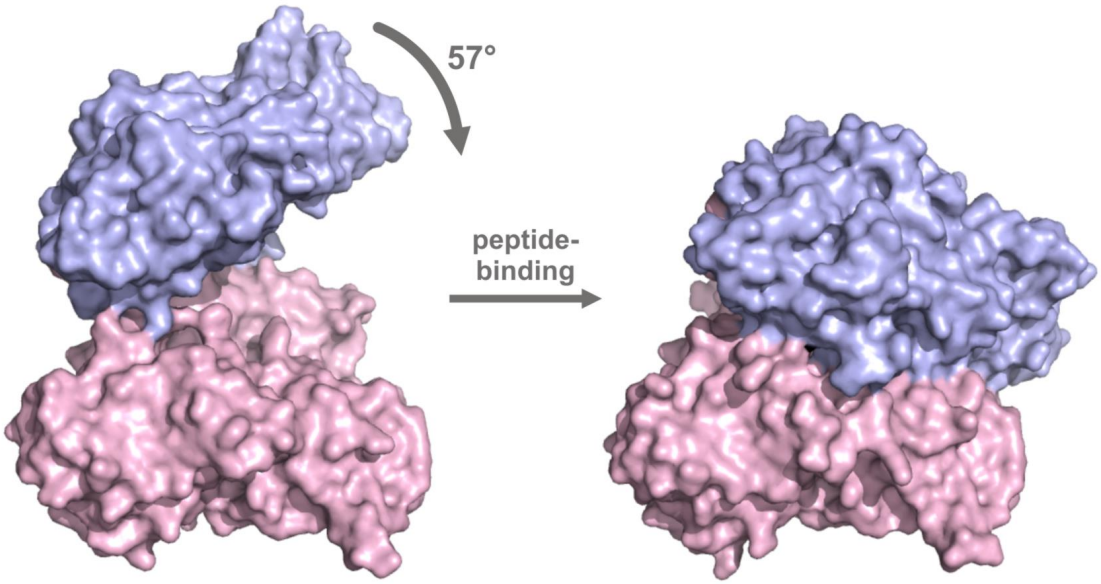




*inactive E461A variant

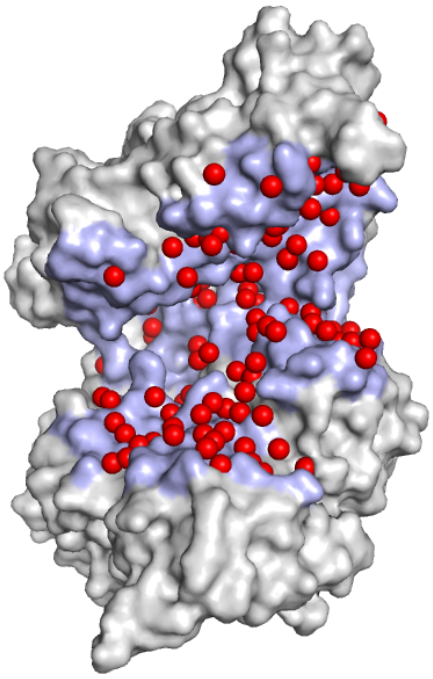
Bezerra *et al.*, *PNAS* **109**:6525-6530, 2012

Domain Motion upon Peptide Binding



Total buried surface area (protein + ligand): 3500 Å^2

Release of Water as Entropy Source

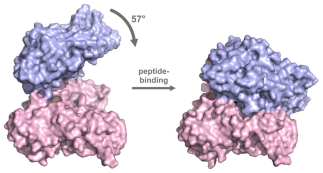


"Entropy Reservoir"

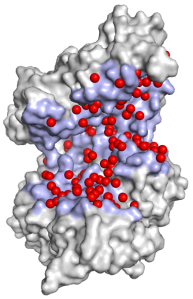
- about 60 ordered water molecules are found in the binding cleft
- from ITC: ΔS = 200-400 J K^{-1} mol^{-1}
- literature data: 5-30 released water molecules yield a $\Delta S°_{solv}$ of 100-600 J K^{-1} mol^{-1}

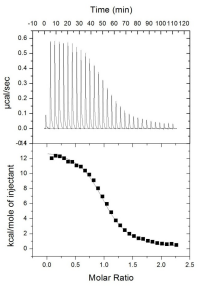
Conclusions

- large conformational change upon peptide binding



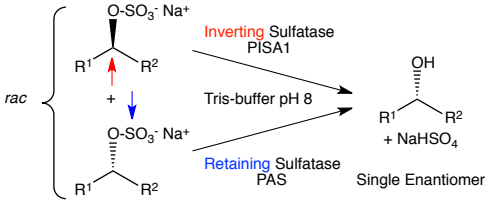
- entropy gain as driving force, fueled by the release of water molecules from the binding cleft

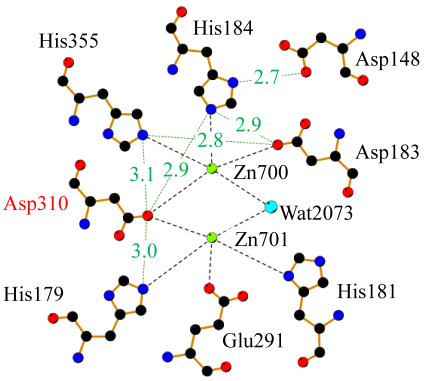




- Unspecificity in enzymes/proteins is favored by a large entropy gain!

Alkylsulfatase from *Pseudomonas aeruginosa*





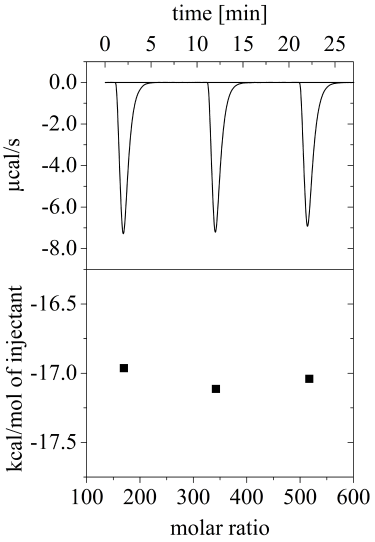
Enzyme Kinetics with ITC

- Only requirement: heat signal (reaction enthalpy)
- Quick (ca. 2 hours)
- Small amounts of material (nM-µM)

$$\text{reaction rate} \approx \frac{dQ}{dt} \quad \text{(thermal power)}$$

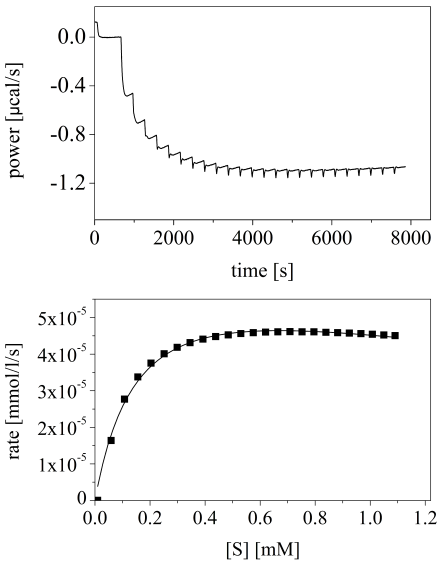
$$\frac{dQ}{dt} = \frac{dP}{dt} \times V \times \Delta H \longrightarrow \text{reaction rate} = \frac{dQ}{V \times \Delta H \times dt}$$

Determination of Reaction Enthalpy and Kinetic Measurement



Syringe: *(R)*-2-octylsulfate, 7 mM

Cell: PISA1, 200 nM



Syringe: *(R)*-2-octylsulfate, 7 mM

Cell: PISA1, 25 nM

Injections:1 x 2 µ; 20 x 10 µL

Catalytic Parameters

1-octylsulfate	K_M [µM]	k_{cat} [min^{-1}]	K_I [µM]
PISA1	660 ± 30	49 ± 4	495 ± 75
SdsA1	27 ± 14	300 ± 106	293 ± 70

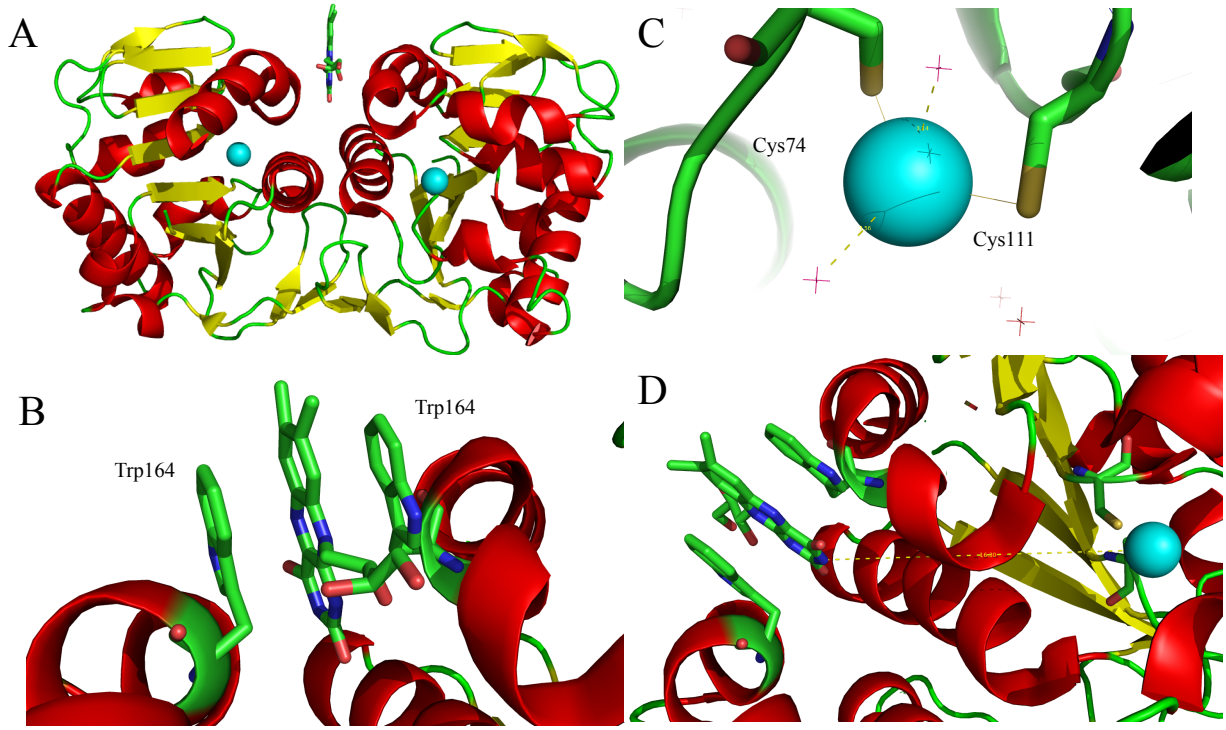
(R)-2-octylsulfate	K_M [µM]	k_{cat} [min^{-1}]	K_I [mM]
PISA1	152 ± 24	263 ± 62	3.8 ± 1.1
SdsA1	361 ± 86	4 ± 0.4	0.92 ± 0.11

⇒ Determination of K_M, k_{cat} and K_I in a single experiment!

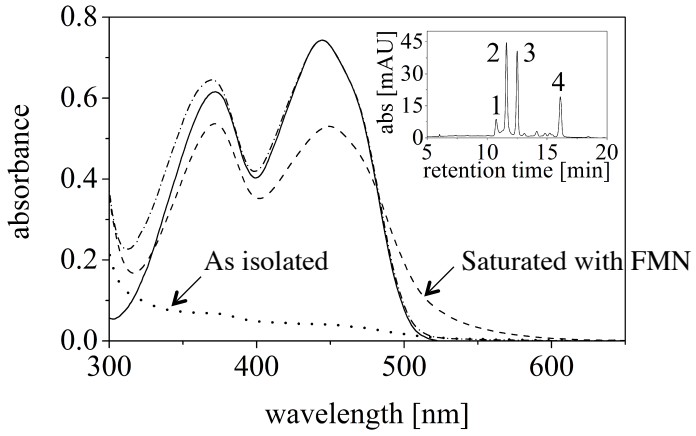
A Putative Protease from *Bacteroides thetaiotaomicron* („ppBat")

- crystal structure solved to 2 Å (MCSG, Zhang *et al* 2008)
- Pfam ID: DJ-1_PfpI ; architecture: 3-layer (αβα) sandwich
- 19,878 kDa molecular mass (homodimer)
- contains 1 FMN, 2 Zn and 2 Ca per dimer
- **1st hydrolase with bound flavin (non-covalently)**

Structural Features of ppBat (3cne)

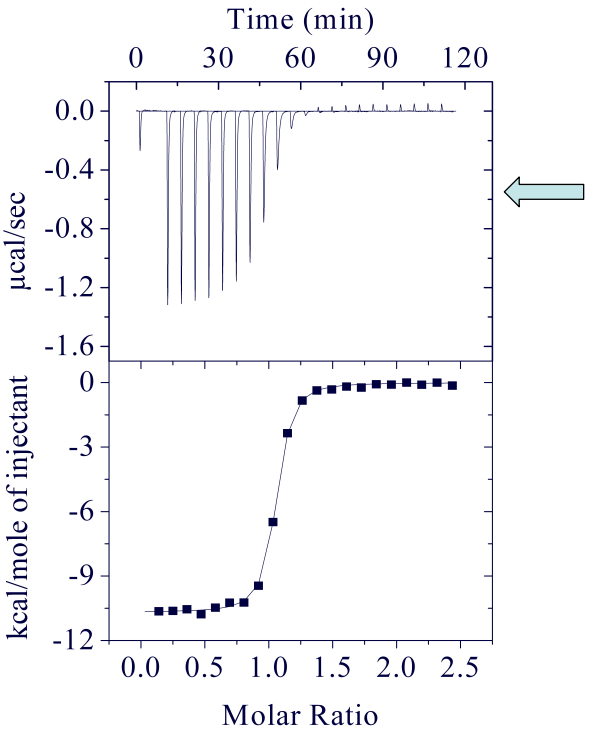


Is ppBat a Flavoprotein?



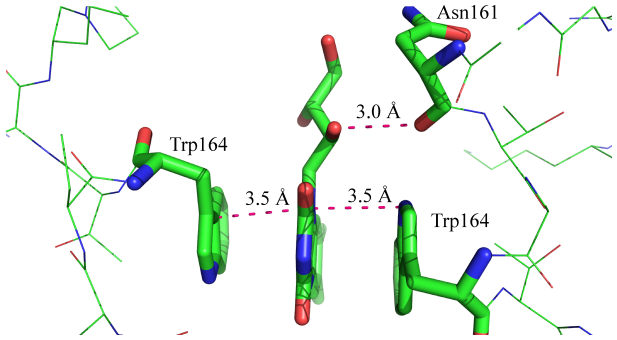
1 = FAD
2 = FMN
3 = riboflavin
4 = lumichrome

Determination of Dissociation Constants by ITC



Flavin ligand	K_d (nM)
lumichrome	272 ± 61 nM
riboflavin	74 ± 5 nM
5-deaza-riboflavin	84 ± 5 nM
iso-riboflavin	96 ± 2 nM
8-amino-riboflavin	227 ± 26 nM
2'-deoxy-riboflavin	487 ± 3 nM
FMN	487 ± 19 nM
FAD	801 ± 82 nM

The Role of Trp164 in Flavin Binding

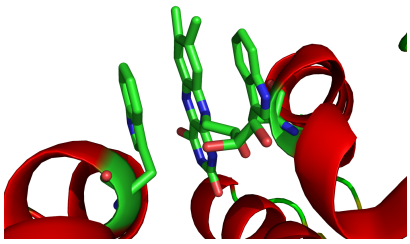


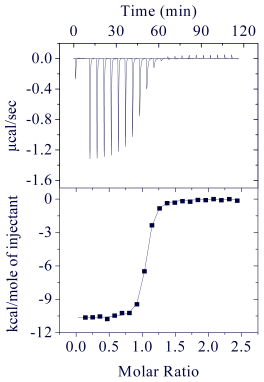
Ligand	W164F	W164A
riboflavin	1190 ± 150 nM (16-fold higher)	No binding
FMN	8460 ± 400 nM (17-fold higher)	No binding

π-stacking interaction essential for flavin binding

Conclusions

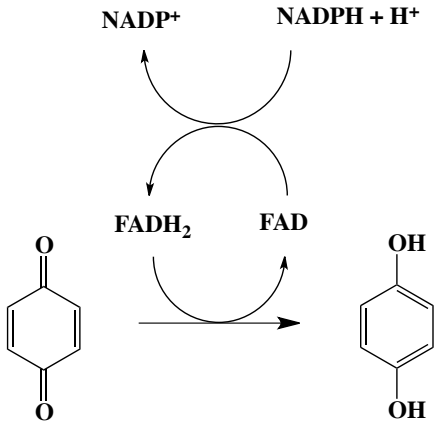
- ppBat “flavin binding site” is unspecific and atypical
- Flavin binding is mainly π-stacking interaction of aromatic ring systems
- Flavin binding not required for protein dimerisation
- That everything in biology has a purpose is a human misconception!





Human NAD(P)H:quinone oxidoreductase (NQO1)

- NAD(P)H:quinone oxidoreductase-1
- Quinone → hydroquinone



Single Nucleotide Polymorphisms

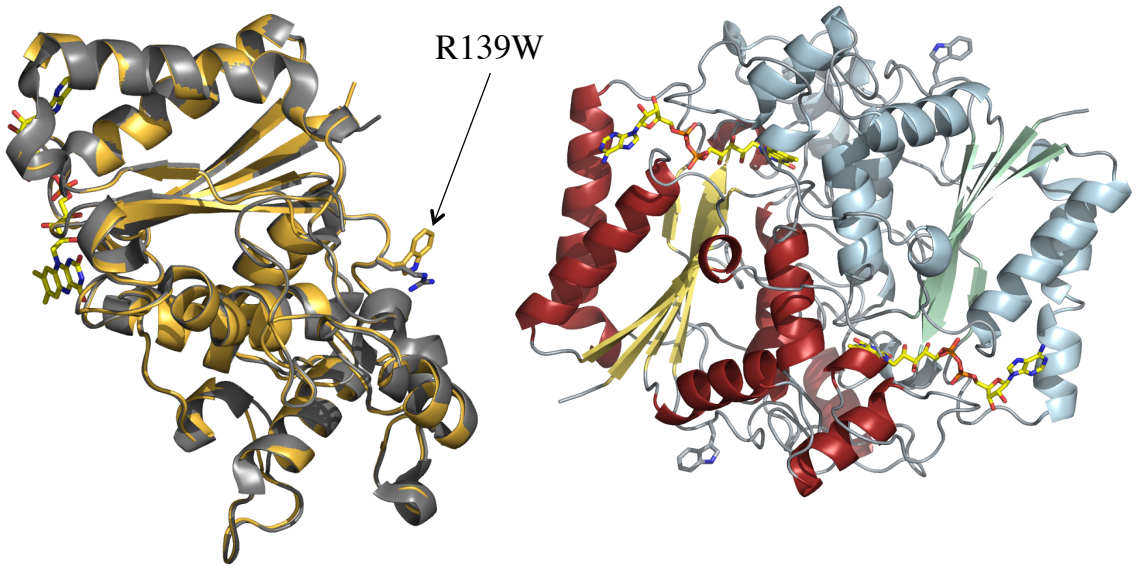
- 609 C>T
  - NQO1*2
  - NQO1 P187S
  - Proline → Serine

- Frequency between 4 and 20 %
- Increased risk for specific cancers
- Increased toxicity of benzene
- Lowered survival rates with cancer

- 465 C>T
  - NQO1*3
  - NQO1 R139W
  - Arginine → Tryptophan

- Found in some cancer lines
- Involvement of the variant in tumour development unknown

Structure of the R139W Variant

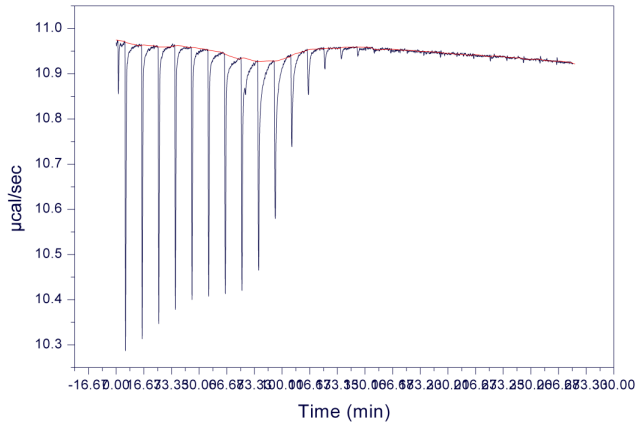


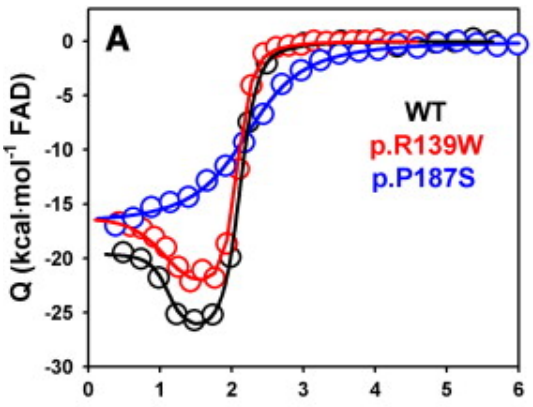
NQO1 R139W

- Behaves (mostly) like NQO1 WT
- Similar UV-visible absorption spectrum
- Similar enzymatic properties
- Similar crystal structure
- Similar 1D and 2D-NMR spectrum (N15-labelled protein)
- But…

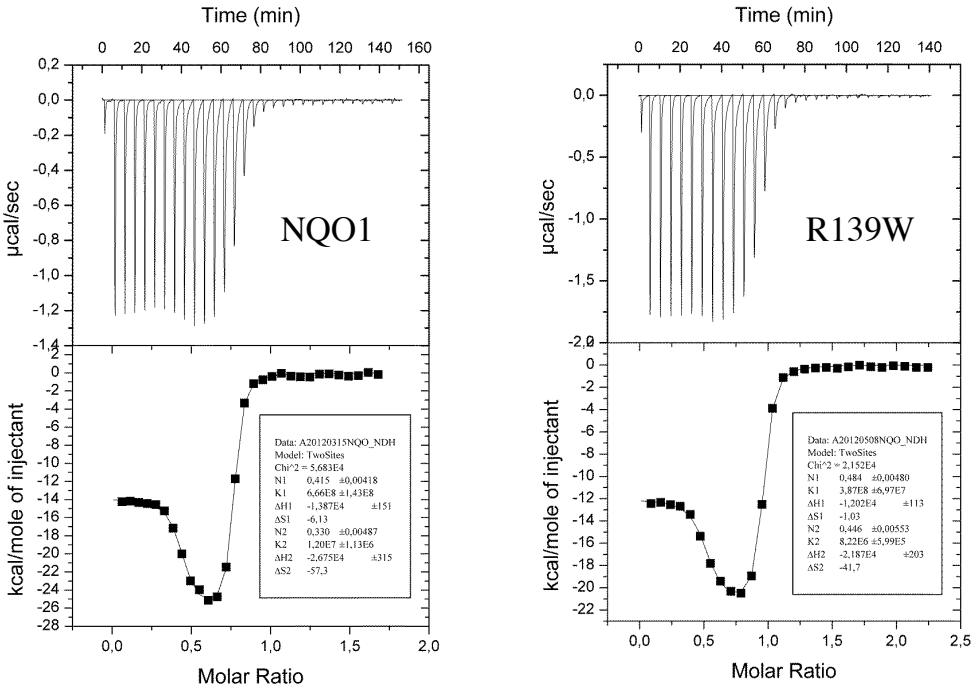
NQO1 – ITC

- Pey AL, Megarity CF, Timson DJ. FAD binding overcomes defects in activity and stability displayed by cancer-associated variants of human NQO1. *Biochim Biophys Acta.* 2014 Nov;1842(11):2163-73.





Titration of Apo-Proteins with FAD



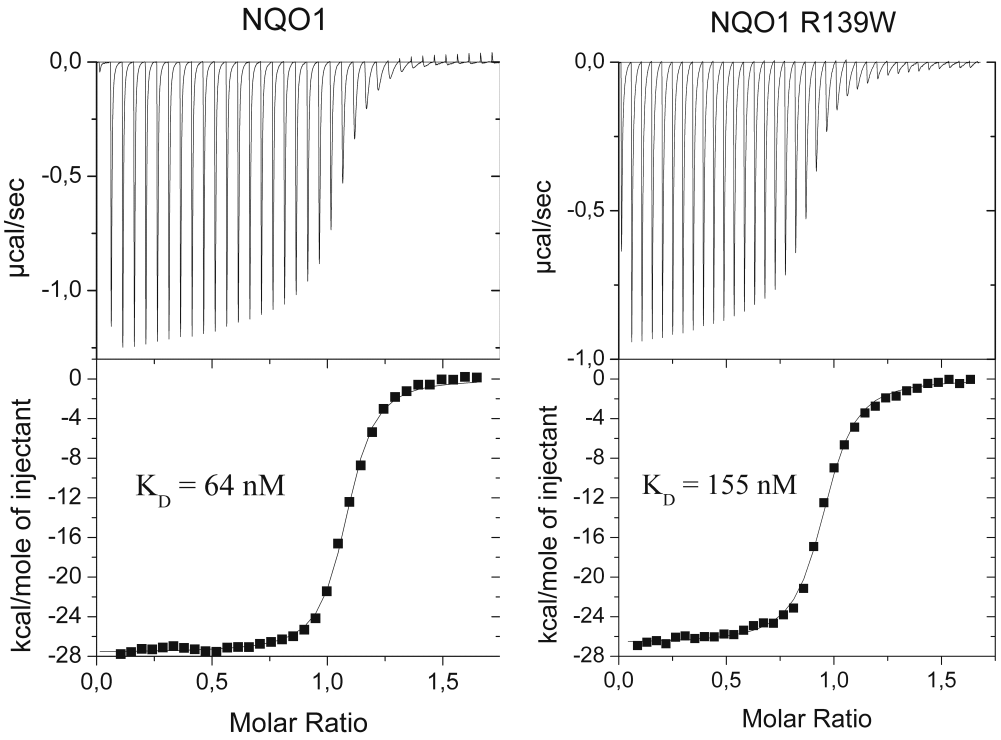
- Fit possible to „two binding site model“
- Protein precipitated in cell!

Summary of ITC Measurements

- FAD cofactor was injected into apo-protein solution
- N values were quite variable
- Apo-protein solution in the sample cell precipitated (same apo-protein solution on ice remained clear!)
- Apo-protein sensitive to shearing forces?

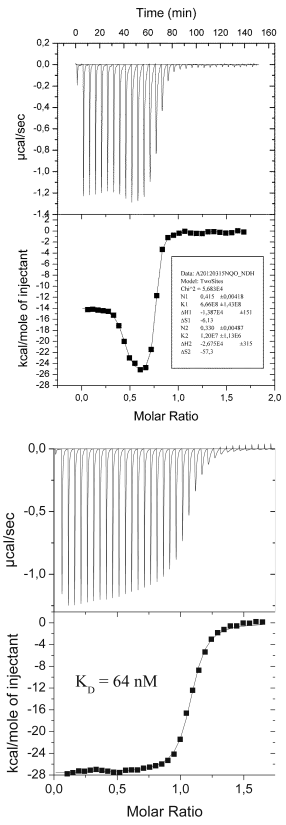
Titration experiments were repeated by titration of FAD with apo-protein

Titration of FAD with Apo-Proteins

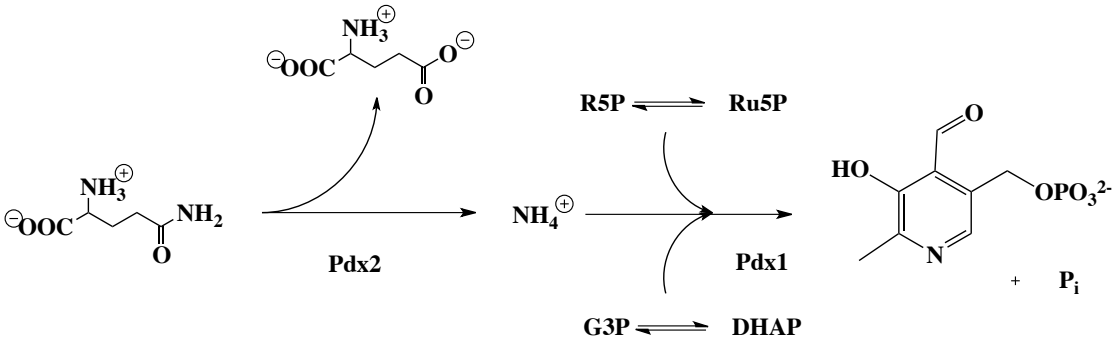


Conclusions

- Unstable samples (proteins): Order matters!
- Consult structure to identify correct ligand binding model



Biosynthesis of Pyridoxal 5'-phosphate (PLP)



L-glutamine

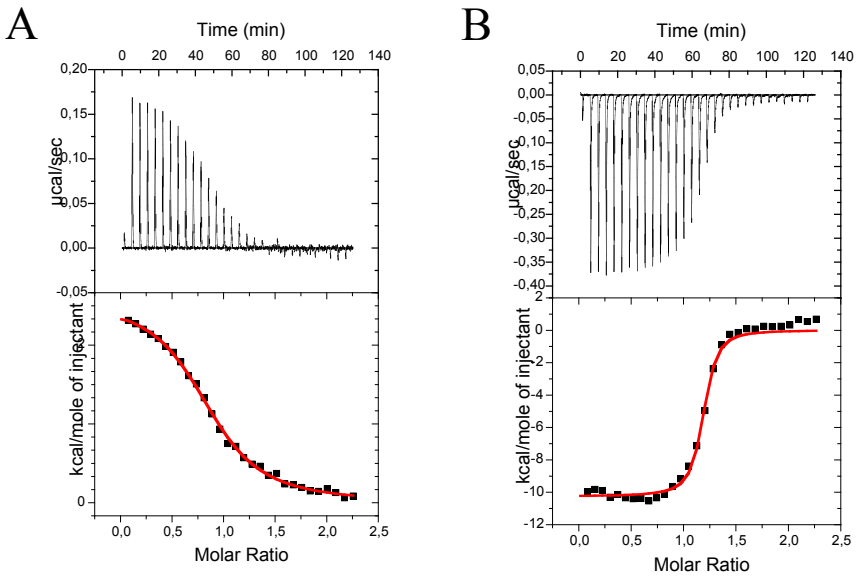
pyridoxal 5'-phosphate

Pdx1 = pyridoxal 5'-phosphate synthase
Pdx2 = L-glutaminase

- Pdx2 (L-glutaminase) activity requires the presence of Pdx1

⇨ Pdx1 and Pdx2 form a protein complex!

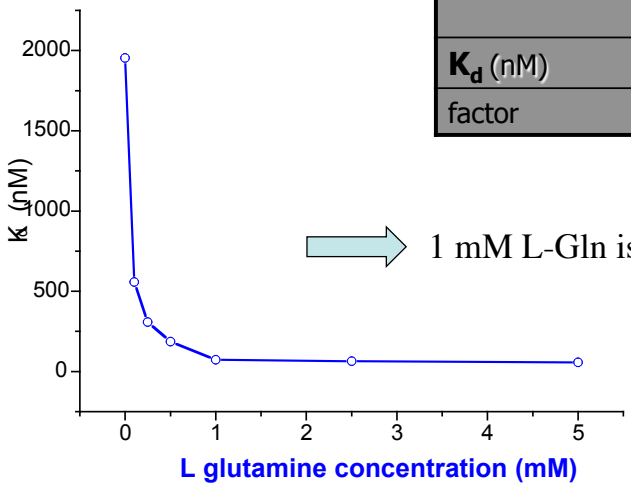
ITC in the Absence and Presence of L-glutamine



T = 25 °C 0 mM L-glutamine 1 mM L-glutamine

Dependence of K_d on L-Gln Concentration

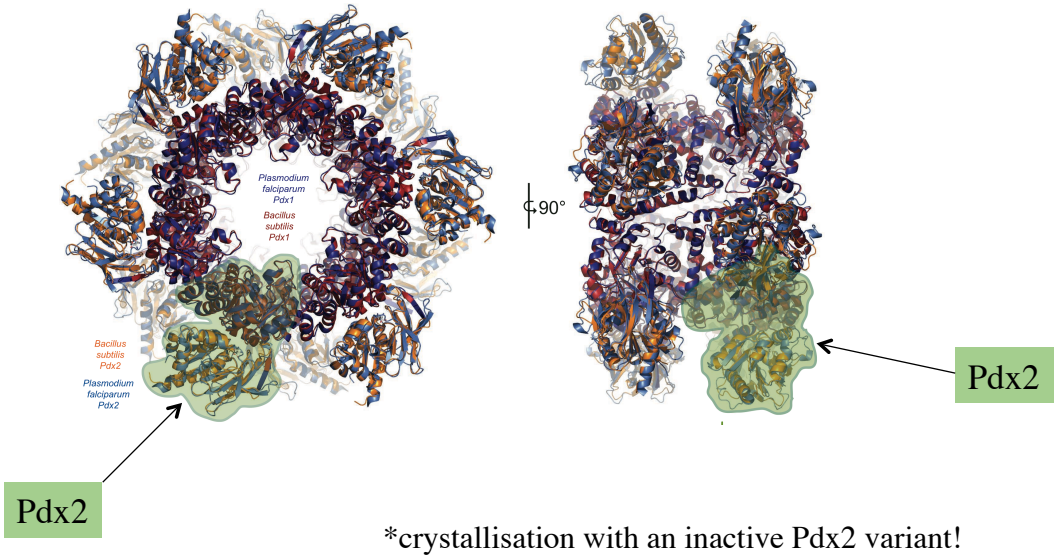
	0 mM L-Gln	1 mM L-Gln
K_d (nM)	1954±322	69±38
factor	1	1/30



1 mM L-Gln is enough to stabilize the interaction

Structure of the Ternary Complex: Pdx1/Pdx2/L-Glutamine*

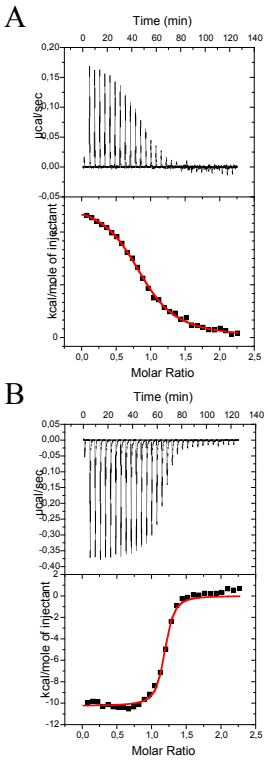
- Pdx1 forms two hexameric rings stacked upon each other ("double donut")
- Pdx2 binds to each Pdx1 at the periphery of the ring structure
- L-glutamine binds near the heterodimer interface



*crystallisation with an inactive Pdx2 variant!

Conclusions

- L-glutamine affects binding affinity of Pdx1 & Pdx2
- L-glutamine changes the forces involved in complex formation



General Conclusions

- ITC is a perfect method to characterize binding processes, if the "system" behaves nicely (lots of stable and soluble material….) - **full thermodynamic profile**
- It is worthwhile spending time on **finding optimal conditions** for your system (buffer, ionic strength, pH, detergents, temperature, order of titration, concentrations, instrument parameters, etc.)
- In ideal cases combined with structural information (**analysis of structure-function relationships**) – Keep an eye water!
- **Understand the processes that are responsible for heat changes**!