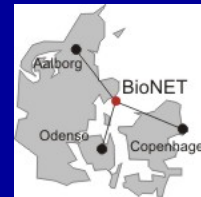


# Protein-protein interactions



+



Daniel Otzen

Department of Molecular Biology

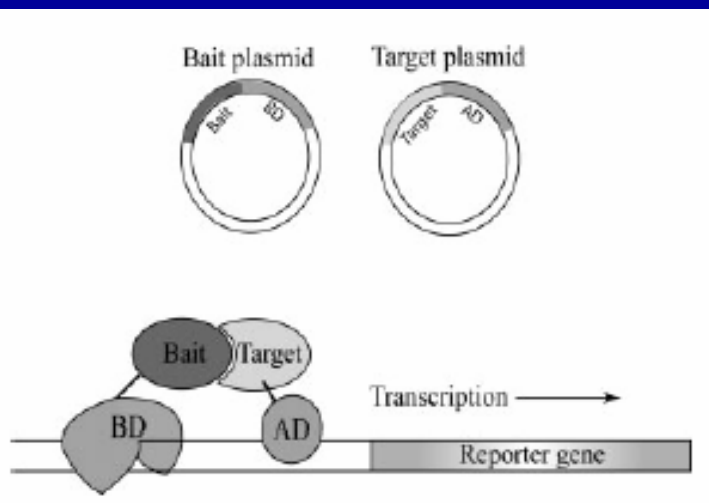
Aarhus University

# Overview

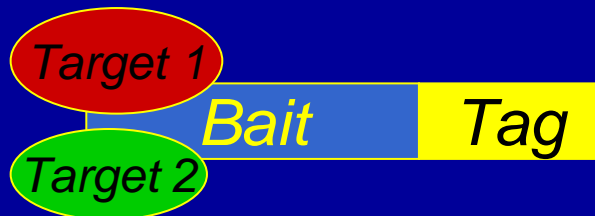
- Ways of getting large-scale information on protein-protein interactions:
  - Yeast two-hybrid system
  - TAP-MS
- Characterizing interactions in depth
  - Confocal microscopy
  - Co-immunoprecipitation
  - Surface plasmon resonance
  - Spectroscopic approaches
  - Isothermal titration calorimetry

# Different ways of identifying protein-protein interactions

## Yeast Two-hybrid system



## Co-affinity purification MS



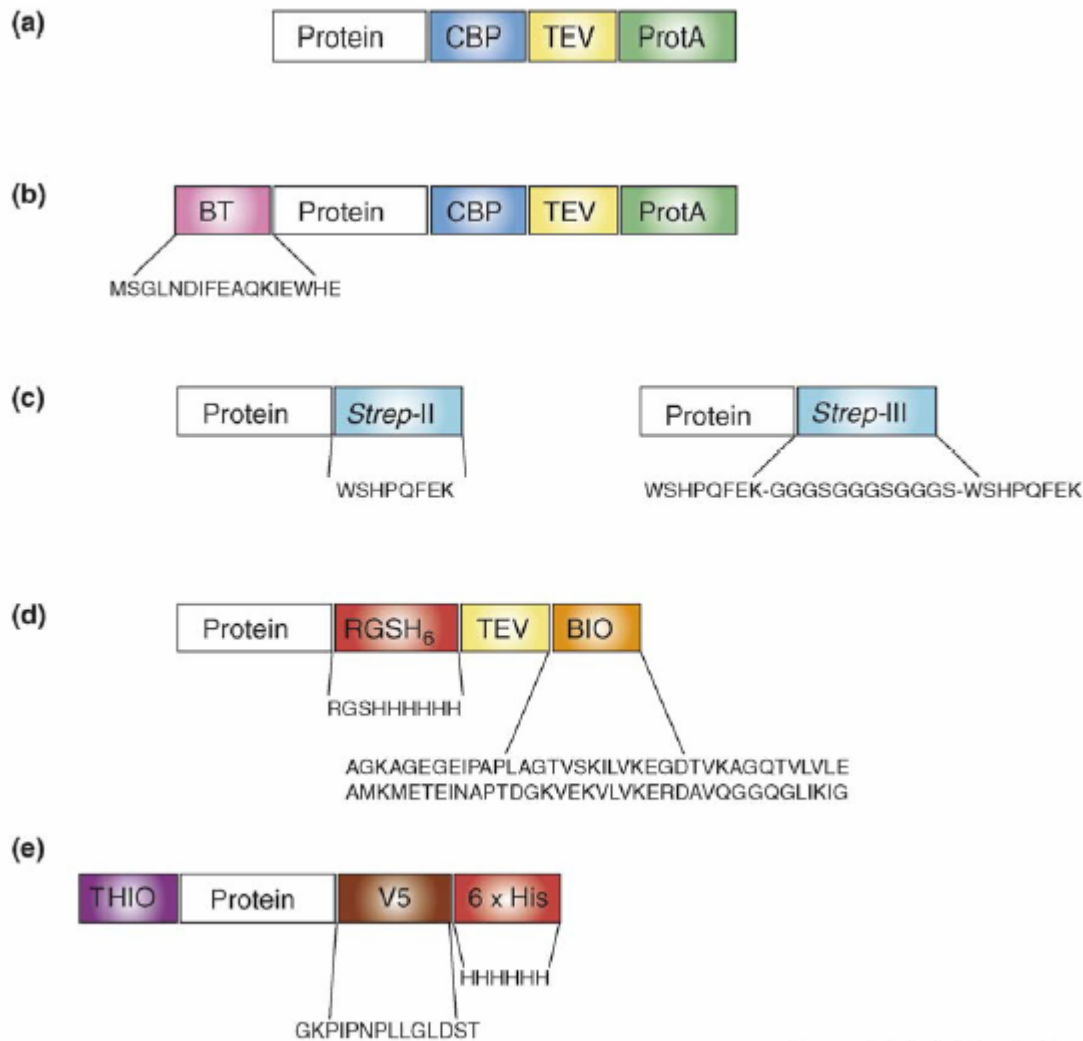
- The binding domain of the bait binds to DNA but only activates transcription if combined with an activator domain from the target sequence.
- Requires all relevant genes to be cloned into the appropriate vector
- Works by signal amplification and so detects transient/weak interactions
- Can also purify complexes by increasingly sensitive co-affinity purification by MS
- Both methods pretty unreliable:
  - 40-80% false negatives
  - 30-60% false positives
- Partly due to enormous sensitivity of MS
- Needs to be validated by other approaches

# Tandem affinity purification-MS



- Express protein in suitable cell line/organism
- Lyse cells and bind via Tag 2
- Cleave off protein via protease
- Bind Bait-Tag1 via Tag 1
- Rinse and elute

# TAPping the baits



Calmodulin-binding peptide, IgG-binding Protein A from *S. aureus*

17-aa BT peptide biotinylated by *E. Coli* holoenzyme synthase

8-aa Strep-II tag binds streptavidin derivative; Strep-III tandem array

75-aa BIO peptide from *P. Shermanii* transcaboxylase is biotinylated

THIO: modified thioredoxin gene

# Variations on TAPs

- Advantages: expression and complex formation *in vivo*
- Disadvantages:
  - Overexpression under artificial conditions may pick up unphysiological targets
  - biased to stable interactions and high-abundance proteins
  - Tags can undergo post-translational modification
  - Tags can "hide" or interfere with binding/localization
  - Overexpressed and misfolded tags attract chaperones

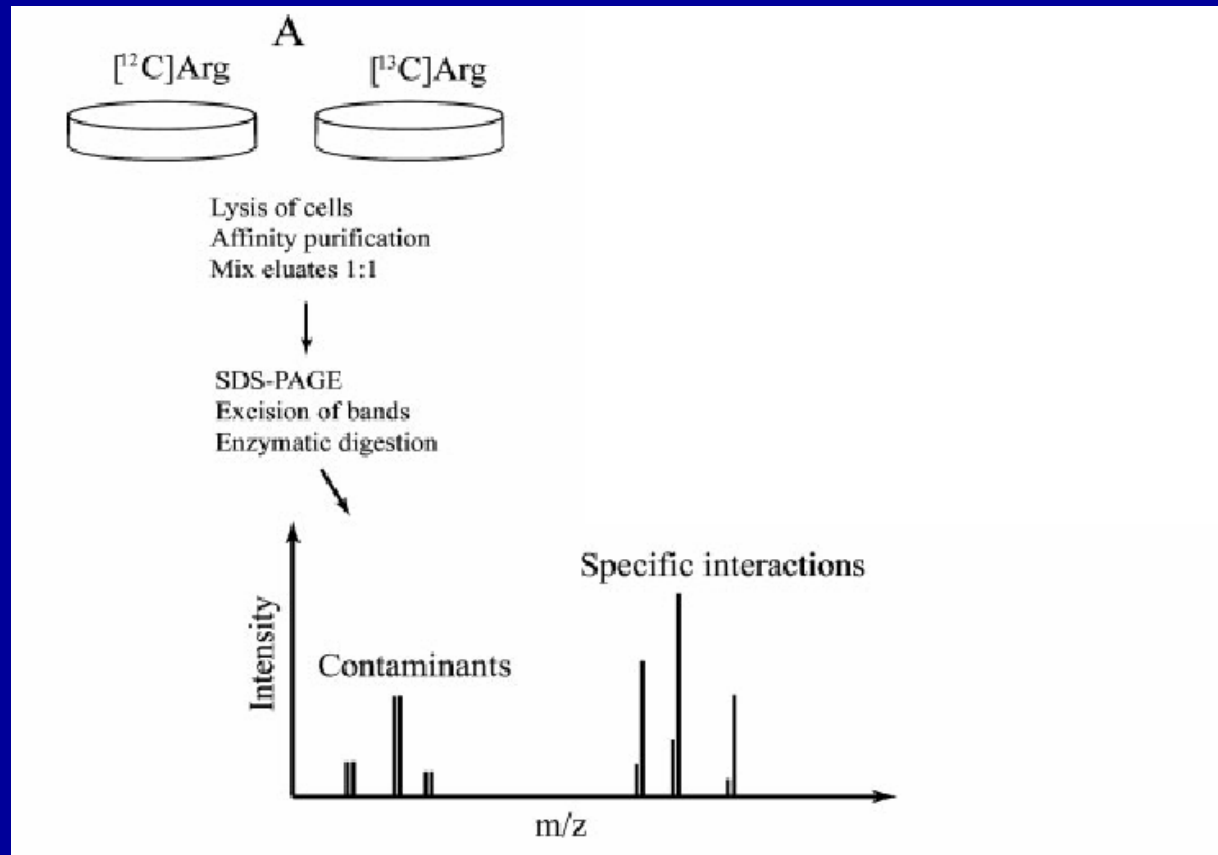
# New TAP prospects

- Can follow up in general by :
  - Replicate purifications to weed out false positives
  - RNA interference of identified components for functional validation
- Single-tag fusion proteins increase yield of low-affinity targets but also increases background.
  - Strep-tag III (binds to modified streptavidin called *Strep-Tactin*) is short tag which interferes minimally with complex formation

# Quantitative proteomics

SILAC: Stable isotope labelling  
with affinity chromatography

ITAC:  
Isotope-coded affinity tag

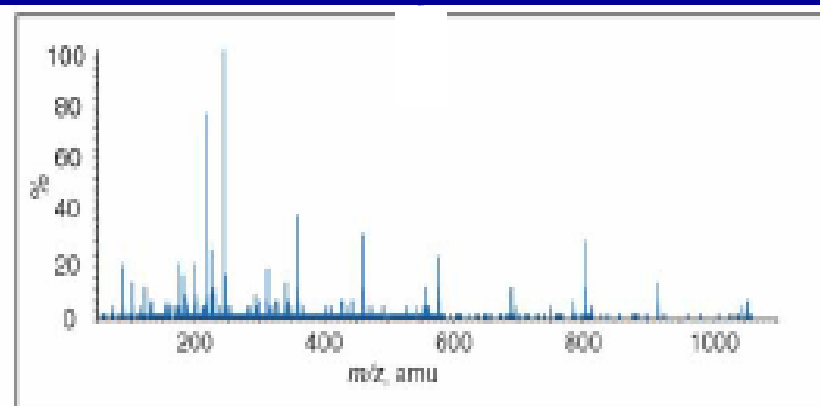
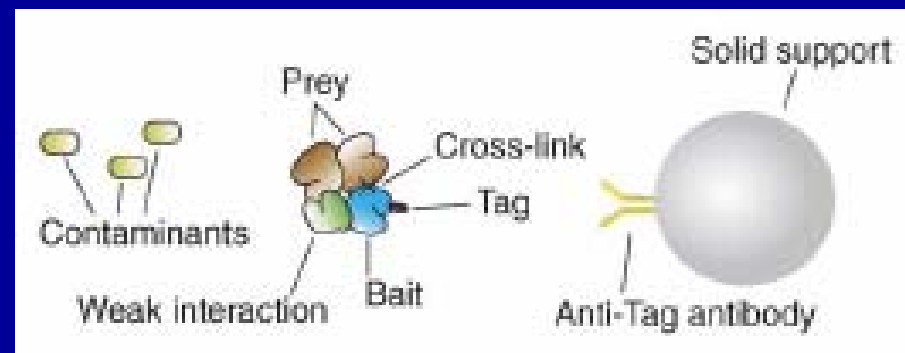
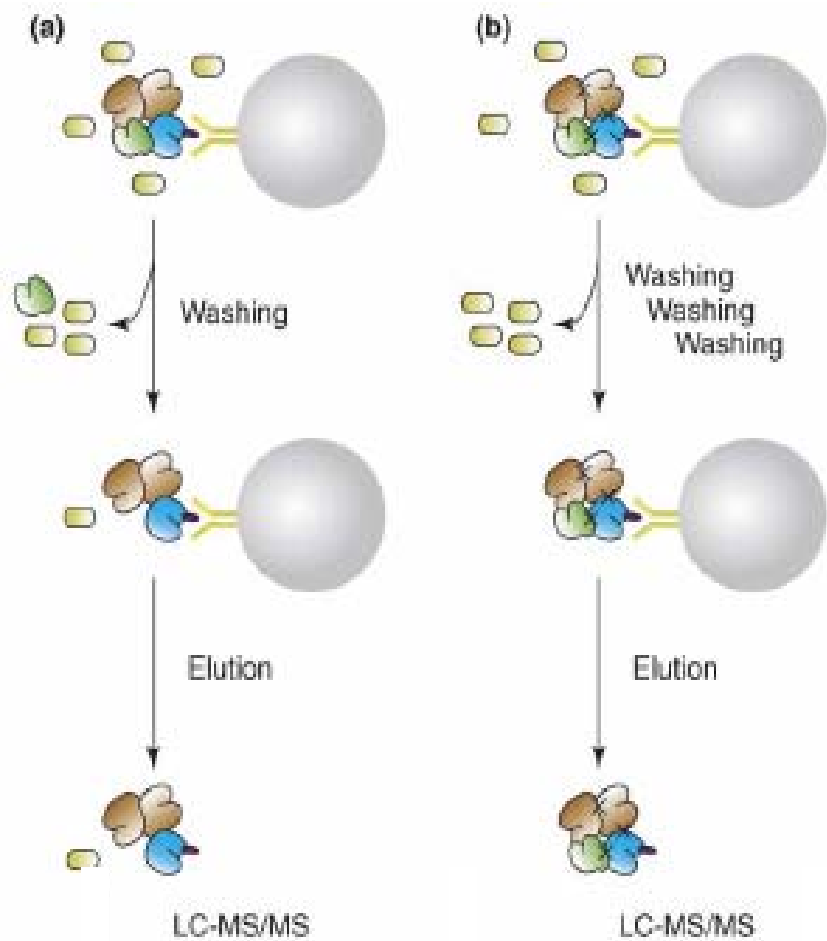


SILAC: metabolic incorporation of aa.  
Stable complexes from heavy cells will  
not mix with stable complexes from light cells

ITAC: light and heavy ICAT reagents  
will not mix between stable complexes

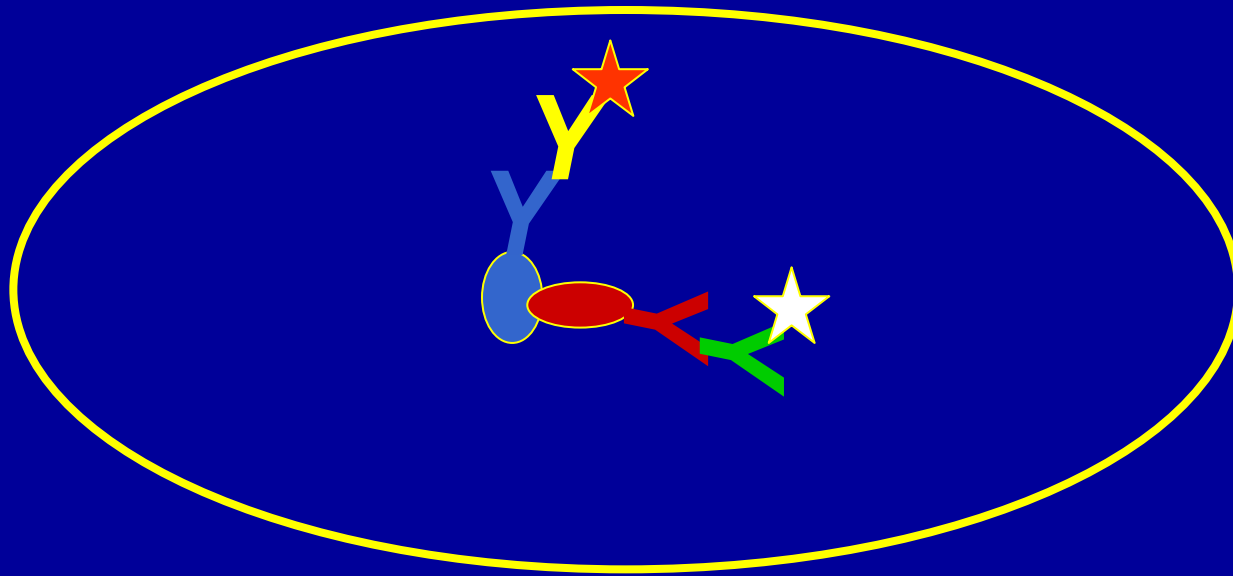
# From stable to transient complexes: chemical crosslinking

- Used for "freeze and capture"
- Formaldehyde: water soluble, membrane-permeable
- Otherwise enormous jungle of different (non)specific crosslinkers
  - Pierce Applications Handbook
- Includes isotopically labelled cross-linkers to generate specific isotope pattern for identification



# How to verify P-P interactions

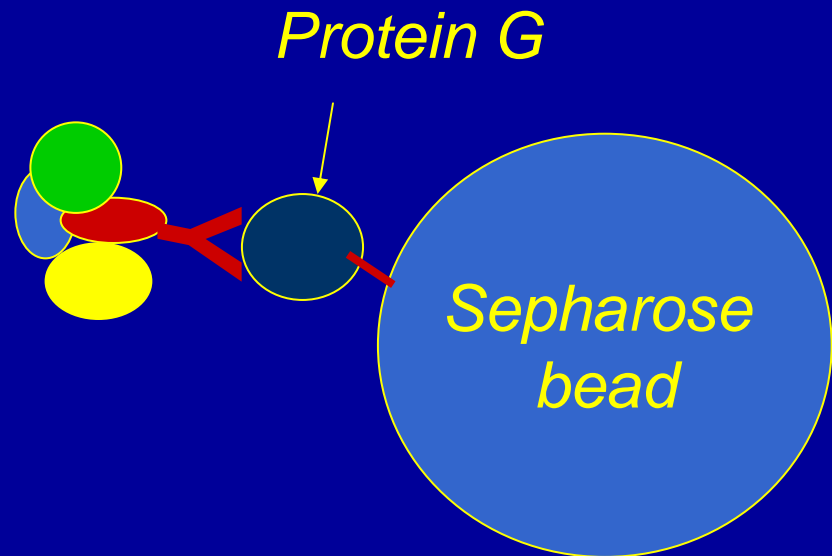
- Confocal microscopy to see that two or more proteins are localized in same part of cell.



- Can be used against endogenous protein if Abs are specific enough.

# Co-immunoprecipitation

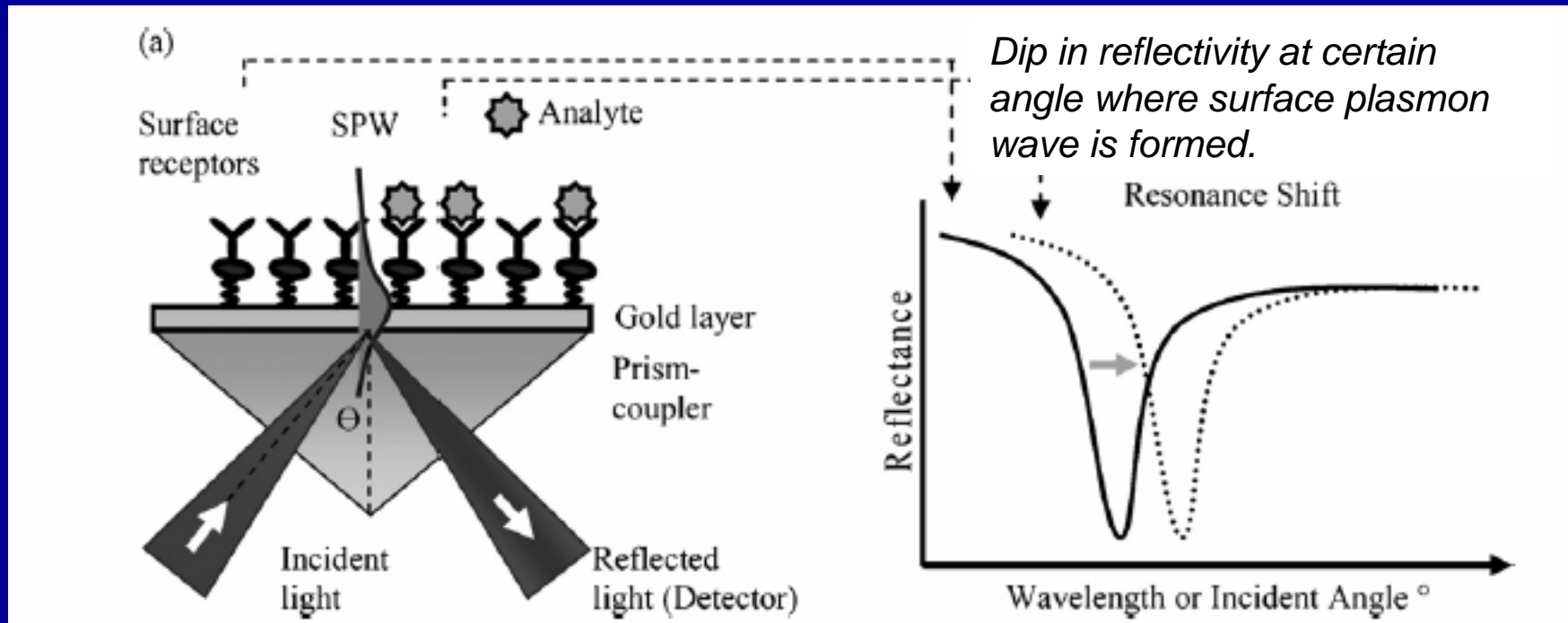
- Significant background.
- Compensate:
- Make antibody  $\Upsilon$  very specific
- Direct against tag
- Elute with specific competing peptides
- Use e.g.  $\Upsilon$  and  $\Upsilon$  in separate experiments



# Direct protein-protein interactions

- Surface plasmon resonance
- Optical spectroscopy and special approaches:
  - SDS-PAGE
  - Analytical ultracentrifugation
  - FRET
- Isothermal titration calorimetry

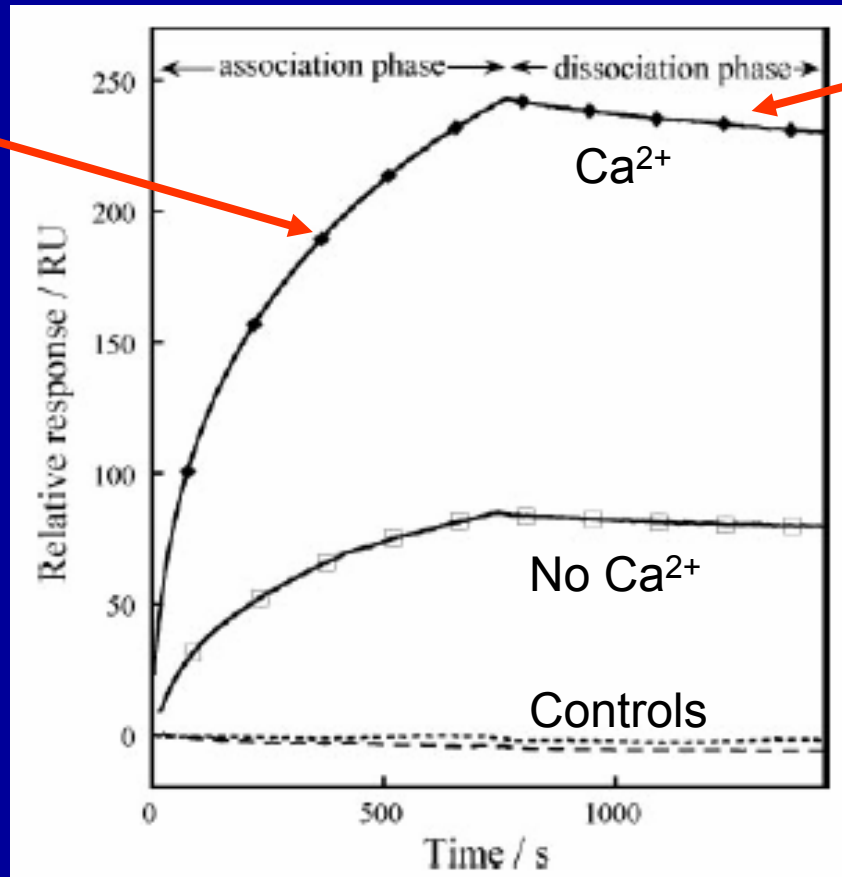
# Surface plasmon resonance



- Real time, label-free, applicable for all molecules
- Kinetic analysis easy:  $K_D = k_{\text{off}}/k_{\text{on}}$
- BUT requires ligand to be surface-immobilized
- Can couple via Cys/Lys/substoichiometric biotin
- Can be used for high-throughput studies

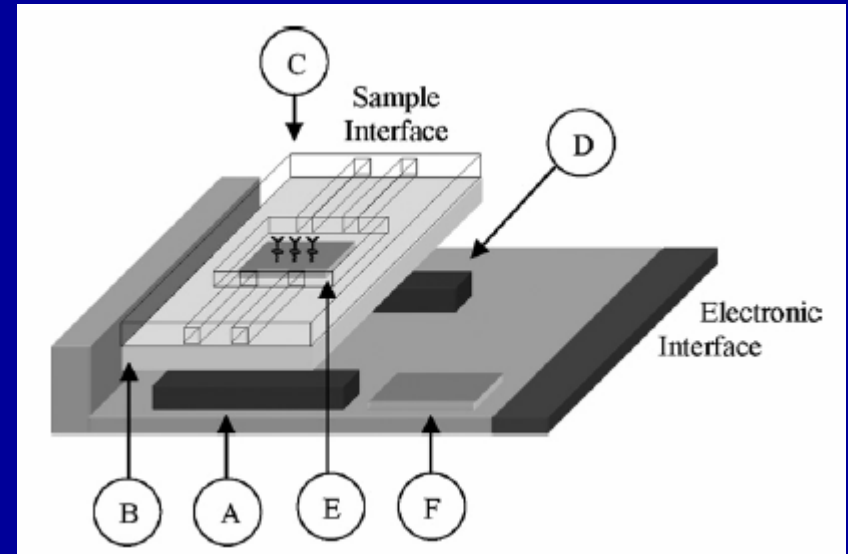
# A typical surface plasmon time profile

Use to get  $k_{ass}$



Use to get  $k_{diss}$

# Lab-on-a-chip

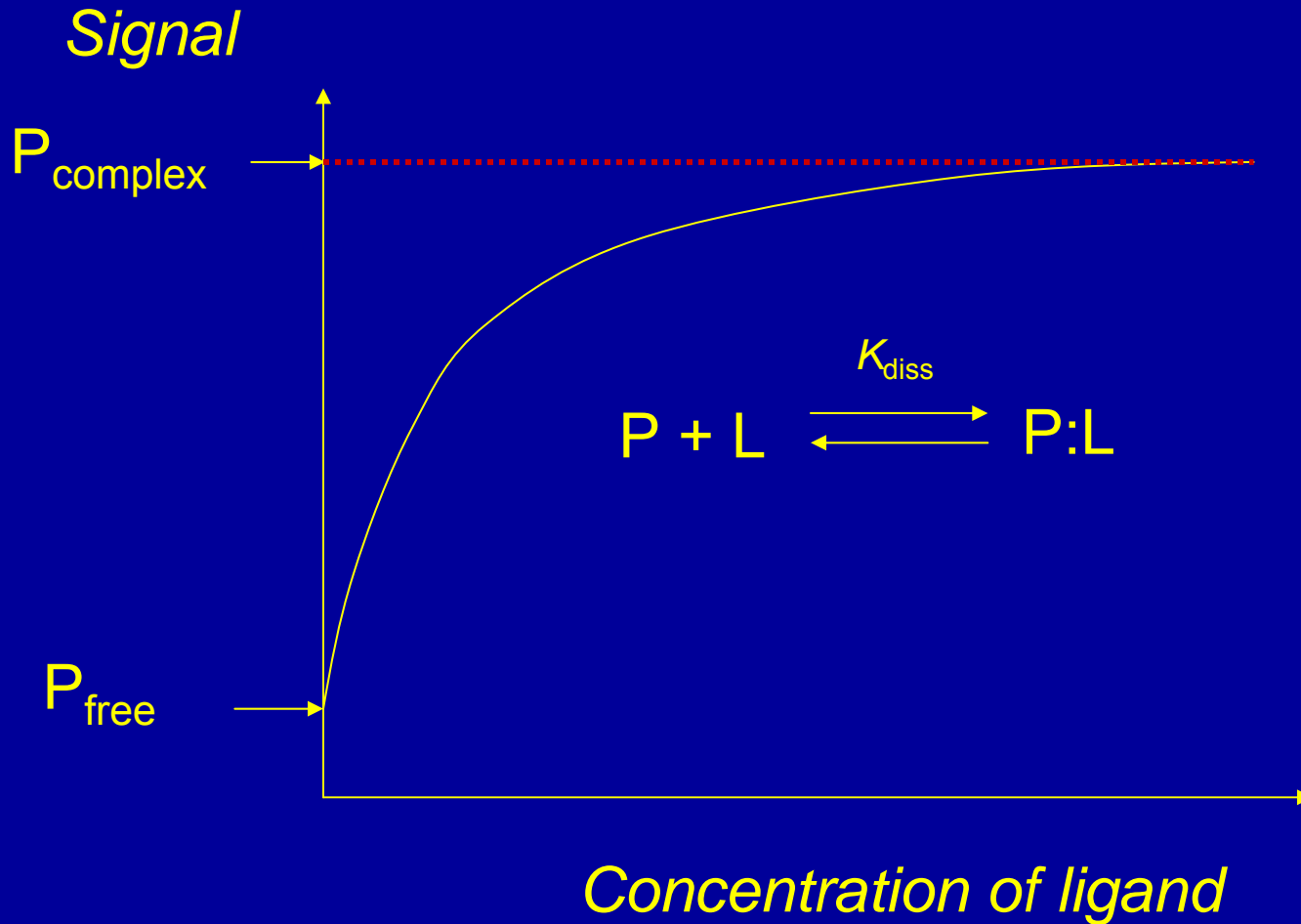


- A (light source) and D (detector): light-emitting diodes or photo-detector arrays
- B (optics): micro-lenses, fiber/waveguides
- C (microfluidics): plastic materials, surface passivation
- E (sensor surface): porous to increase surface area, regenerative surface, nano-particles, chemical patterning
- F (data analysis): Automatic parameter fitting, statistical analysis, integrated temperature control

# Spectroscopic approaches

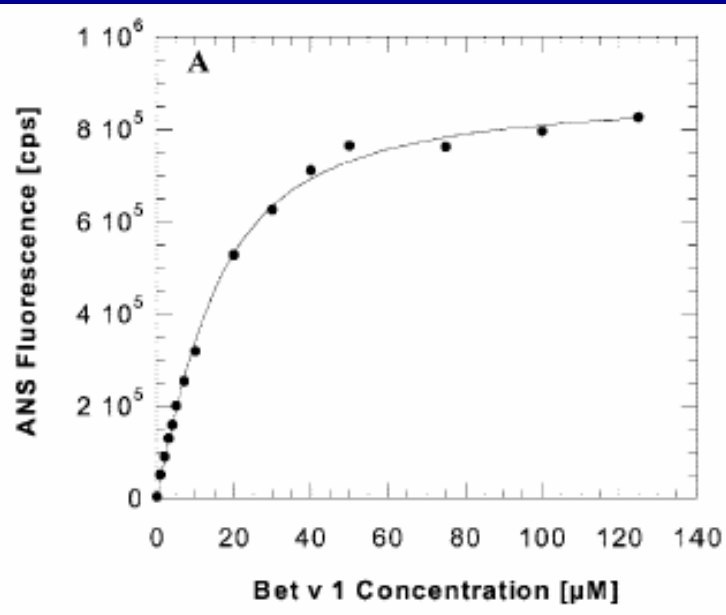
- Identify a spectroscopic handle that changes when a specific complex is formed:
  - Fluorescence intensity
  - Fluorescence polarization (on small peptide)
  - Förster Resonance Energy Transfer (FRET), e.g. involving CFP and YFP
  - Circular dichroism
  - NMR chemical shift/intensity

# Any signal change will do

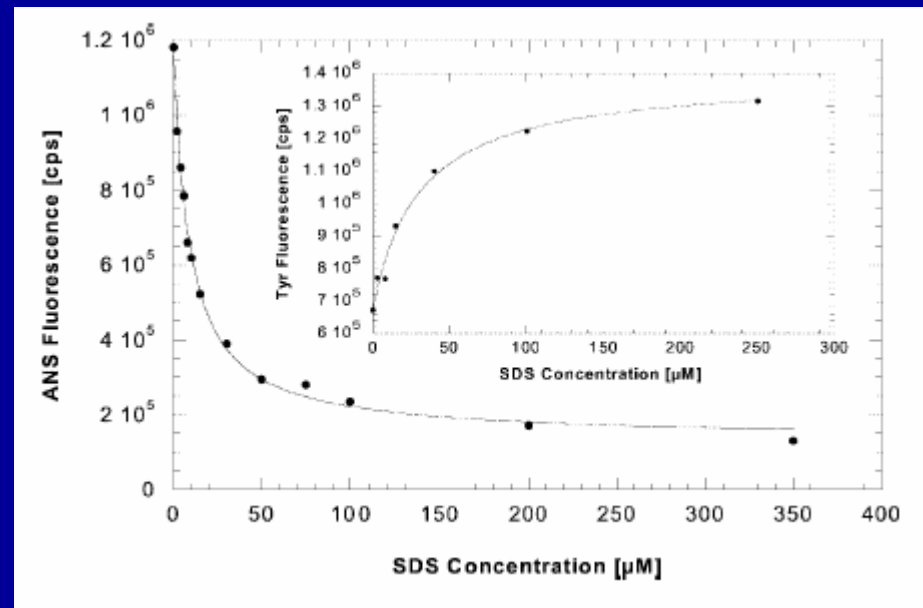


# Direct and indirect measurements

Direct measurement of ANS binding

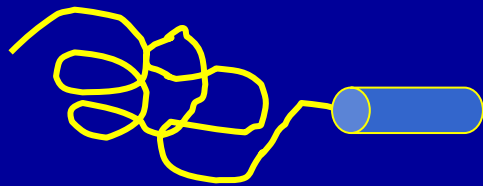
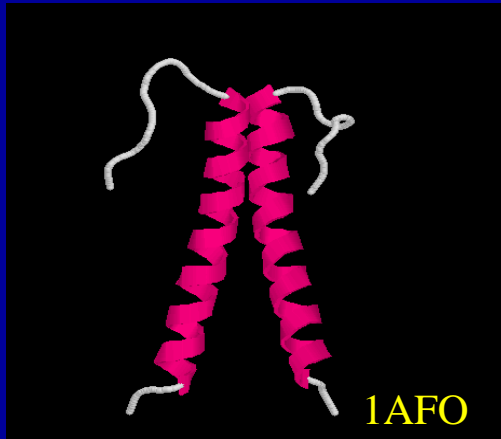


Indirect measurement of other binders by displacement of ANS



Mogensen et al. J. Biol. Chem. 277 (2002), 23684–23692

# Particularly for membrane proteins...

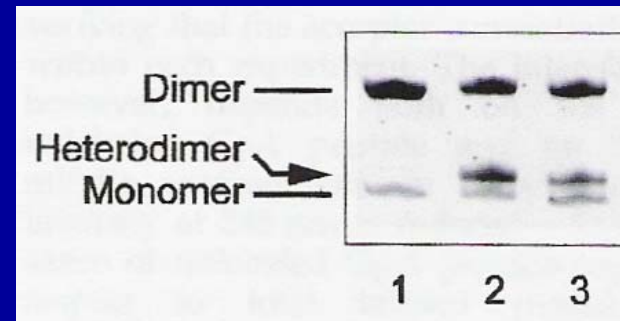


Staph. nuclease TM domain of GIpF  
23 aa

Why use fusion protein?

1. High yields and easy purification in *E. Coli*
2. More sensitive (higher  $\epsilon$ )
3. Technically easier to work with larger protein

SDS-PAGE  
(dimer is SDS-resistant)



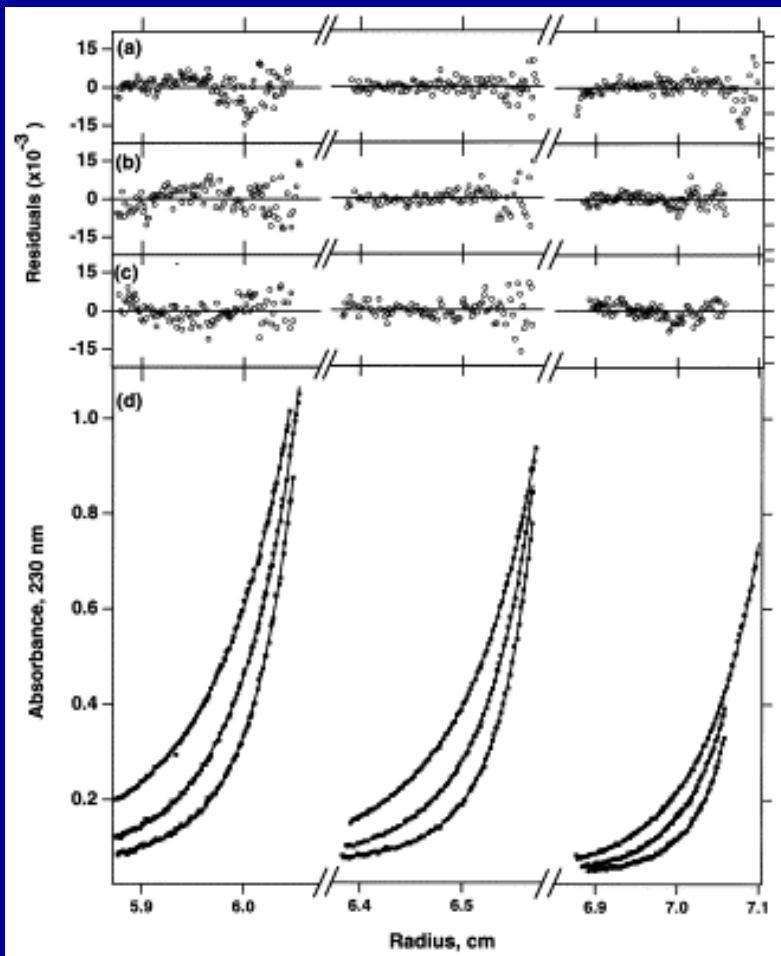
Advantage:

- Quick and easy. Can e.g. quantitate as
- (a) % monomer and dimer (quick scans)
  - (b) Outcompete with TM peptide

Disadvantage:

- Can only use for trends.  
SDS-PAGE destabilizes complex.

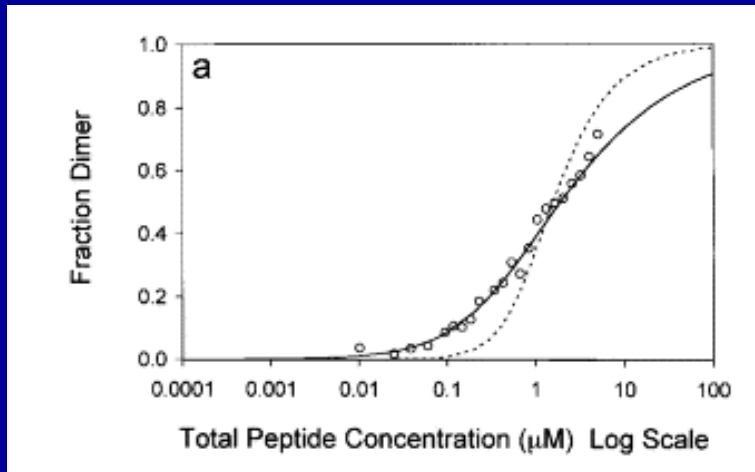
# Analytical ultracentrifugation



- Can be used for both hetero- and homocomplexes
- Also possible for membrane proteins if use non-denaturing non-ionic detergent with neutral buoyancy.
- Equilibria between monomers, dimers and empty micelles must be attained within normal centrifugation run (16-28 hours).

Fleming et al. (1997), *J. Mol. Biol.* 272, 266-275.

By reducing [P]/[L], can shift the equilibrium towards monomer and fit data to simple binding curve.



$$K_d = \frac{[M]^2}{[D]}$$

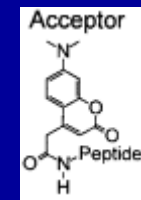
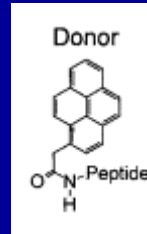
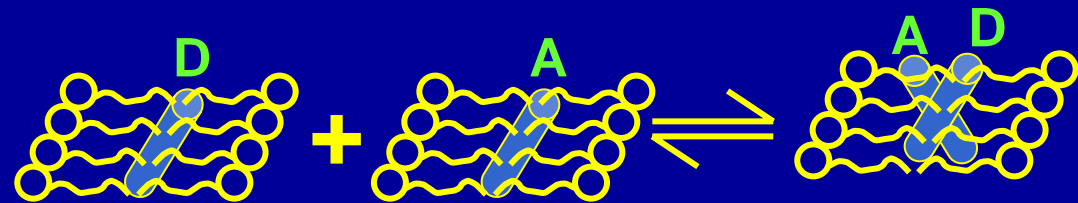
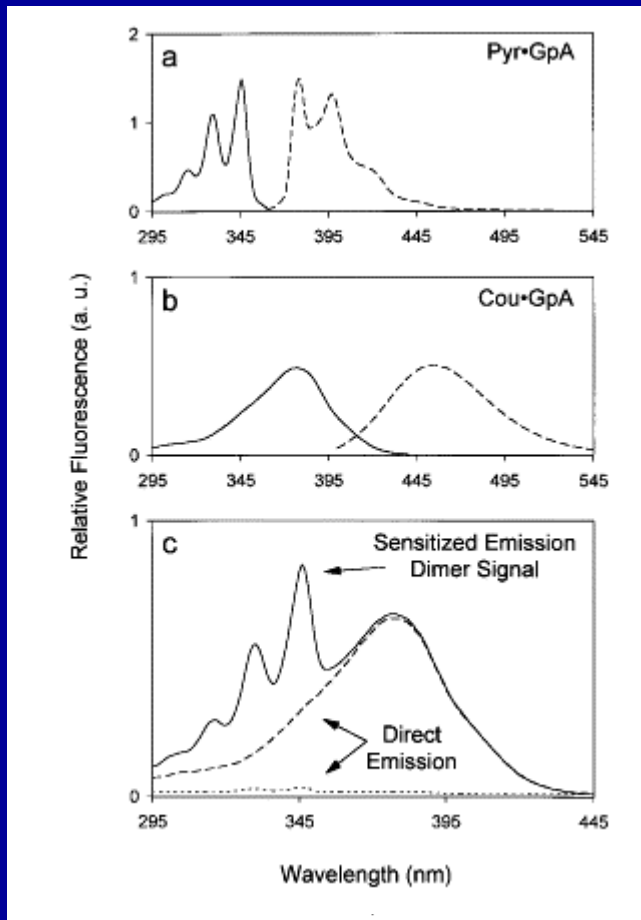
### Serial dilution

Micelle environment	$K_d$ ( $\mu\text{M}$ )	Excess detergent (mol:mol)
DDMAB <sup>a</sup>	$0.08 \pm 0.04$	260,000
DPC <sup>a</sup>	$0.16 \pm 0.08$	150,000
SDS (mM)		
25	$\sim 10$	2200-3100
20	3.6	5600
15	2.9	5100
10	3.0	3300

Fisher et al. (1999) *J. Mol. Biol.* 293, 639-651

# Förster resonance energy transfer

High sensitivity, good for low dissociation constants



Emission 500 nm  
(donor emission minimal)

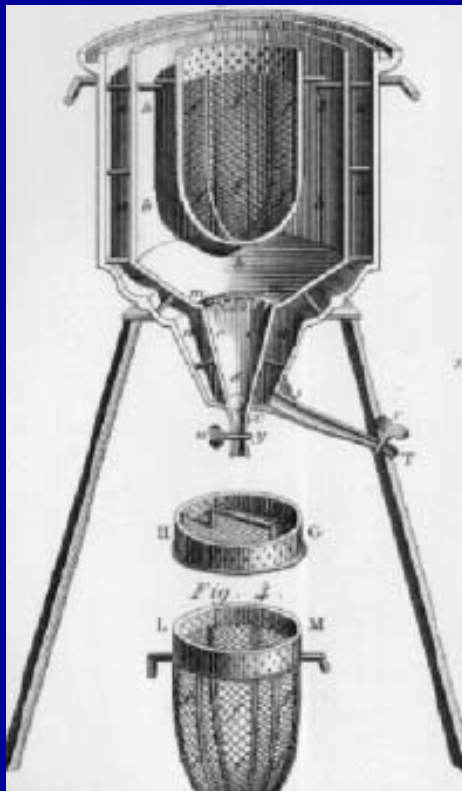
$$E(r) = \frac{r^6}{R_o^6 + r^6}$$

$R_o = 60 \text{ \AA}$

# Isothermal titration calorimetry

*All physical, chemical or biological processes are accompanied by changes in heat.*

*However, early instruments were not that sensitive...*

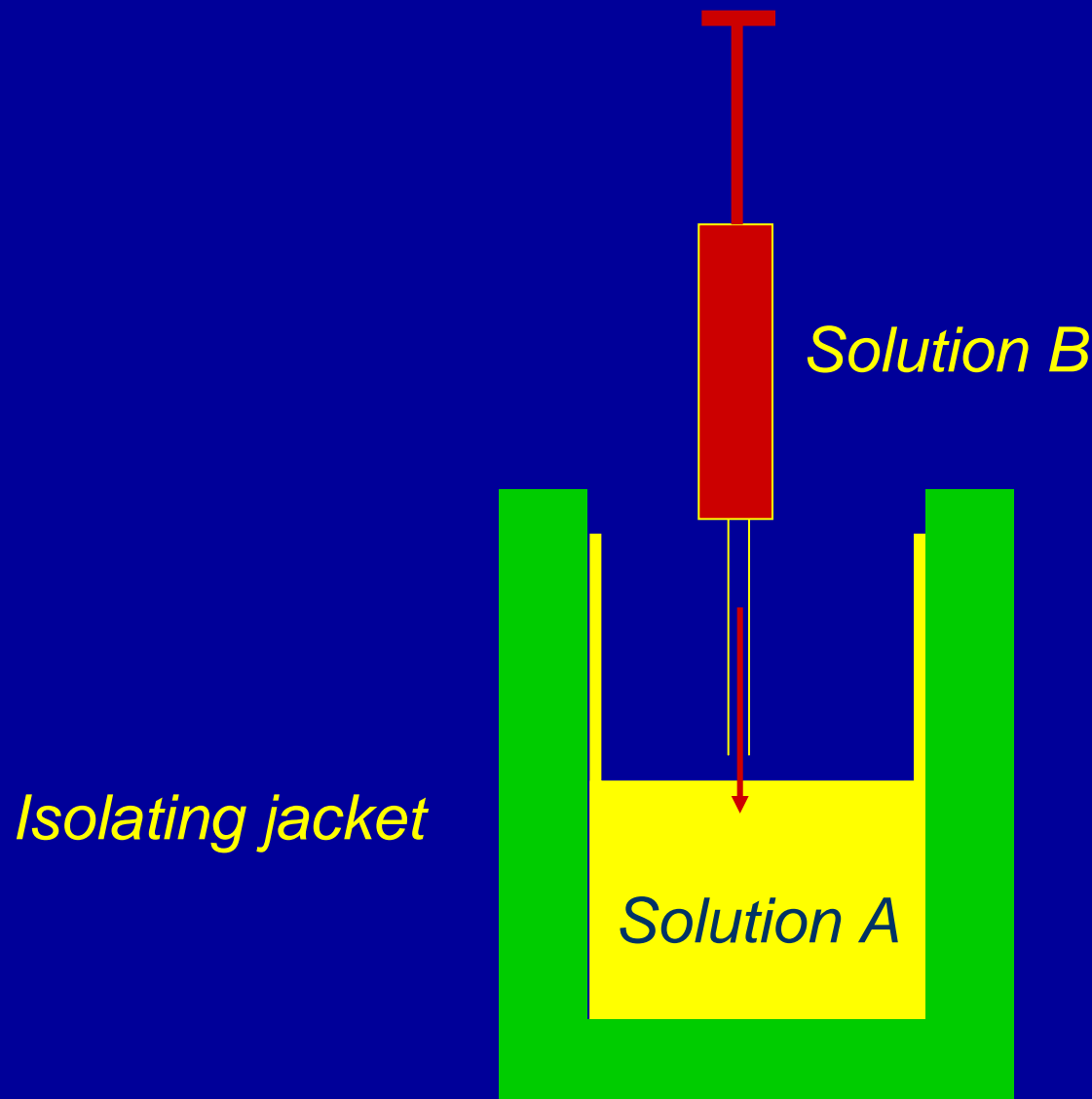


*Lavoisier and Laplace 1780:  
Heat of combustion measured as amount of  
water melted inside the inner ice jacket.  
Used to measure metabolic rate of a guinea  
pig.*

*Best done on days when the temperature  
was just above 0°C....*

# The modern MicroCal VP-ITC

Directly measures heat flow necessary to keep temperature in cell constant within  $10^{-6}$  °C

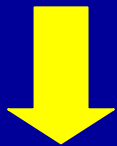


- Easy to handle
- 0.2-1.4 ml cell
- 10-100  $\mu$ M protein
- Stirring paddle to ensure rapid mixing

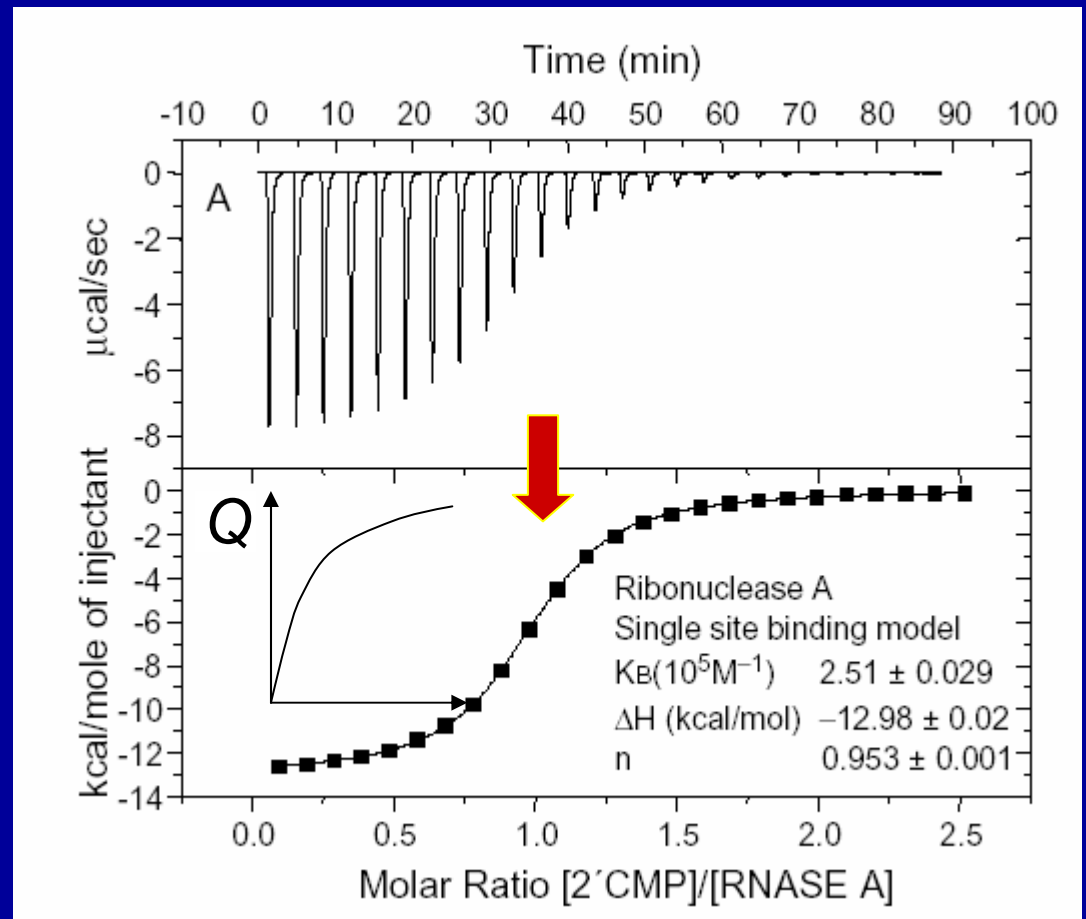
# What information does ITC provide?

A single experiment will usually yield:

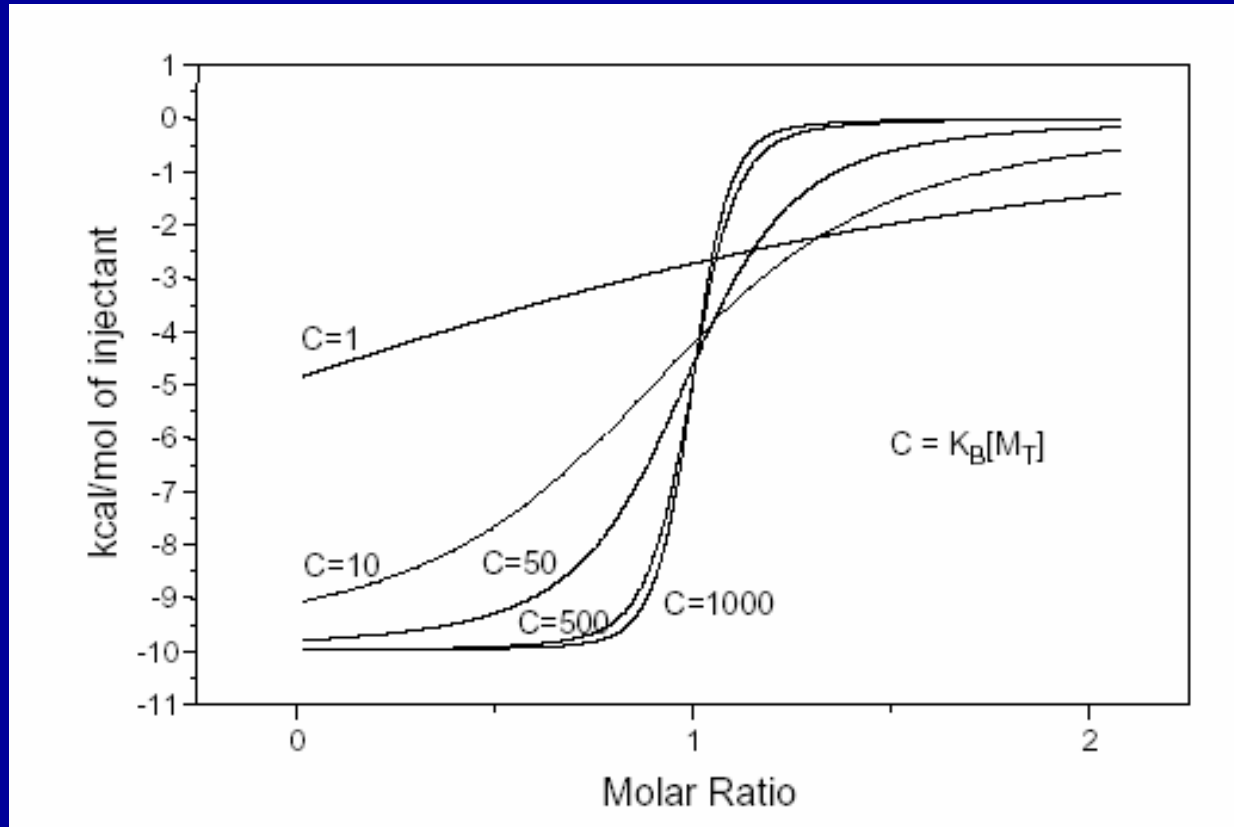
- Enthalpy of binding ( $\Delta H$ , heat of interaction)
- Stoichiometry of binding  $n$
- Affinity of binding ( $K_{\text{diss}} = 1/K_{\text{ass}}$ )



- Free energy of binding  $\Delta G = -RT \cdot \ln(K_{\text{diss}})$
- Entropy of binding  $\Delta S$



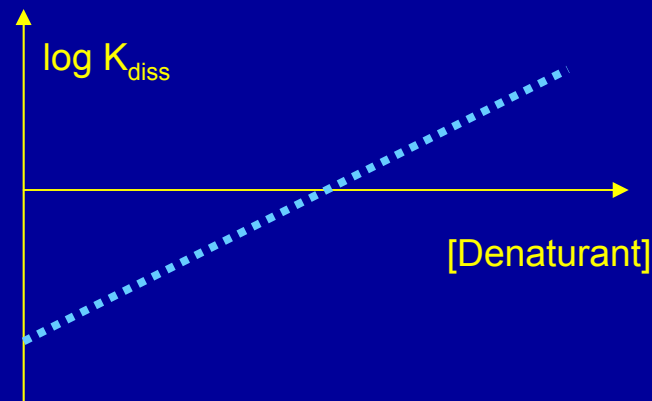
# Which concentration range?



- $C = [\text{protein}]/K_{\text{diss}}$
- Should be around 10-100 for best results.
- Too high affinity gives a step-function rather than a titration
- Too low affinity gives a very shallow curve

# How to get around high affinity

- Extrapolate from unphysiological /harsh conditions
  - High temperatures
  - high denaturant concentrations
  - Other pH-values



- Carry out displacement experiments (displace weak ligand by stronger)

# What can we use this for?

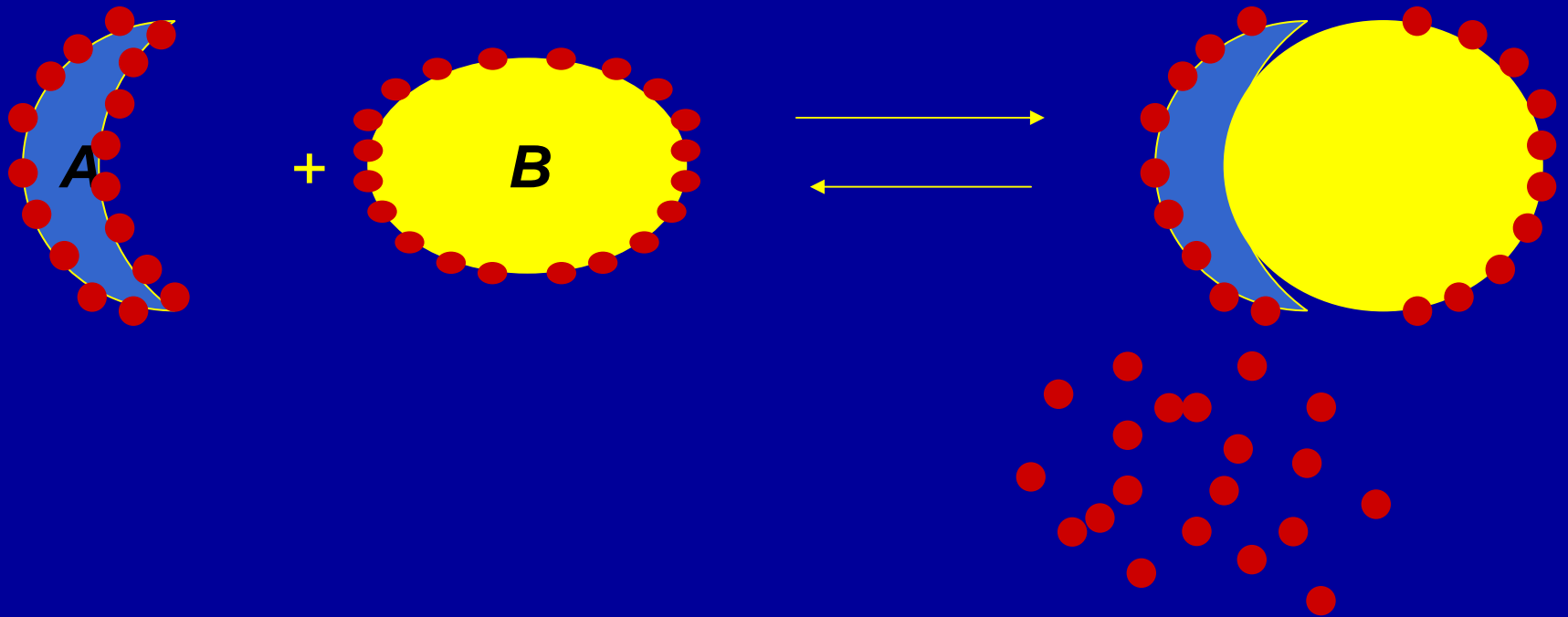
- Understand the forces of binding
  - Energetics of bond formation ( $\Delta H$ )
  - Restriction in conformational freedom ( $\Delta S_{\text{conf}}$ )
  - Release/uptake of water and ions ( $\Delta S_{\text{solvent}}$ )
- And there's more:
- By carrying out ITC experiments at different temperatures, can also obtain the specific heat capacity  $\Delta C_p$ :
- $$\Delta C_p = \Delta c_{np} * \Delta ASA_{np} + \Delta c_p * \Delta ASA_p$$

# Using the different parameters

- Binding free energy  $\Delta G$  describes whether the complex is stable at all
- Balance between "order" ( $\Delta H$ ) and "freedom" ( $\Delta S$ ):  
$$\Delta G = \Delta H - T\Delta S$$
- Increase in order (bond formation) is typically offset by loss of freedom (immobilization): *H-S* compensation
- Same  $\Delta G$  value for two different complexes can be based on different combinations of  $\Delta H$  and  $\Delta S$
- So measure both  $\Delta H$  and  $\Delta S$  if you want to understand your complex...

# ...and understanding them

- ... Remember that you measure *system* changes



Changes in enthalpy:

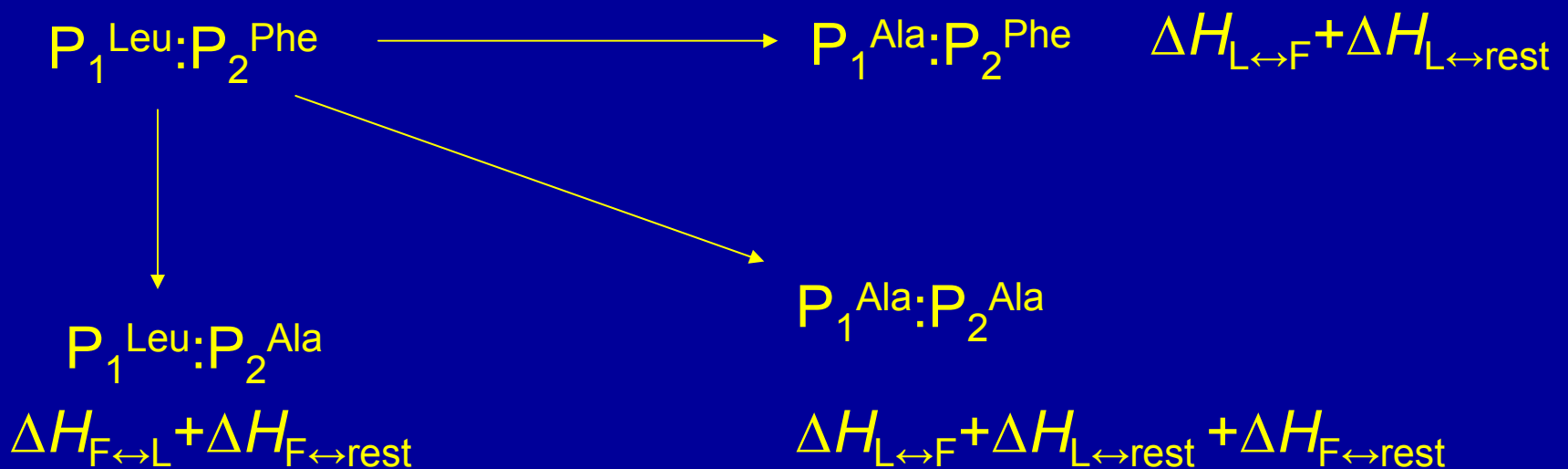
A-water, B-water, B conformation, A-B contacts, water-water

# Homing in on individual interactions

- Rational protein engineering where individual functional groups are removed



- Extend with double mutant cycles



# Can we predict binding energetics?

- $\Delta C_p = \Delta c_{np} * \Delta ASA_{np} + \Delta c_p * \Delta ASA_p$
- $\Delta H_{bind}^{60^\circ C} = \Delta h_{np} * \Delta ASA_{np} + \Delta h_p * \Delta ASA_p$
- $\rightarrow \Delta H_{bind}^t = \Delta H_{bind}^{60^\circ C} + \Delta C_p(t-60^\circ C)$
- But have not taken into account:
  - $\Delta H_{conformation}$
  - $\Delta H_{protonation}$
- $\Delta S = \Delta S_{solvent} + \Delta S_{conf} + \Delta S_{r/t}$
- $\Delta S_{solvent} = \Delta C_p * \ln(T/385K)$
- $\Delta S_{r/t}$  ca.  $-8 \text{ cal mol}^{-1} \text{ K}^{-1}$
- $\Delta S_{conf}$  cannot be calculated directly!

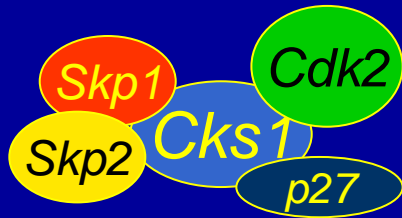
# Case-study: HSV1 Thymidine kinase binding to substrates



- Determine  $\Delta H_{\text{dt}}$  and  $K_{\text{diss}}^{\text{dT}}$  by direct titration
- Determine  $\Delta H_{\text{ATP}}$  and  $K_{\text{diss}}^{\text{ATP}}$  in second step
- Use to calculate  $\Delta G$  and  $\Delta S$  for each step
- Measure  $\Delta H_x$  at different  $t \rightarrow \Delta C_p^x$
- $\Delta C_p^{\text{dT}} = -360$ ;  $\Delta C_p^{\text{ATP}} = -360$ ;  $\Delta C_p^{\text{dT:ATP}} = -510$  cal/K/mol
- Stoichiometry = 0.7-0.8 due to inactive protein
- From  $\Delta S$  and  $\Delta C_p$  calculate  $\Delta S_{\text{solv}}$  (large and favourable) and  $\Delta S_{\text{conf}}$  (large and unfavourable)
  - Due to both freezing of bonds and tightening of TK domains
  - Lousy correspondence between  $\Delta C_p^{\text{measured}}$  and  $\Delta C_p^{\text{calculated}}$  confirm large conformational changes

# Case-study cont'd

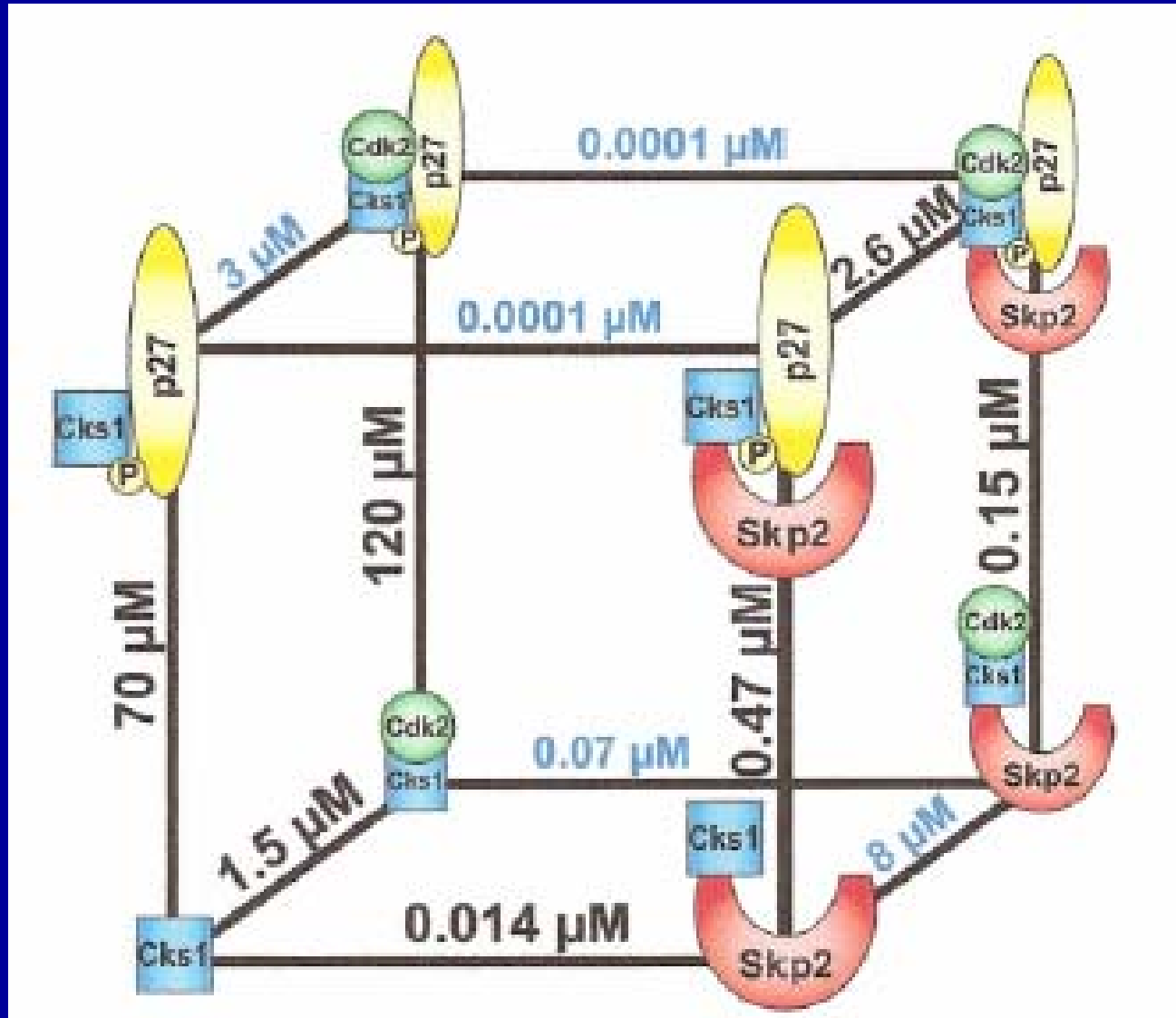
- Mutational analysis gives more information:
- Dead mutation M128F shows 200-fold reduced affinity for dT (increase in  $K_{\text{diss}}^{\text{dT}}$  and less favourable  $\Delta H_{\text{dt}}$  )
- Affinity due to unproductive orientation, since  $T\Delta S$  increases (becomes more favourable) by 15 kcal/mol
- Compensating H58L mutation restores both  $\Delta H$  and  $\Delta S$  but makes TK less broad-spectred!



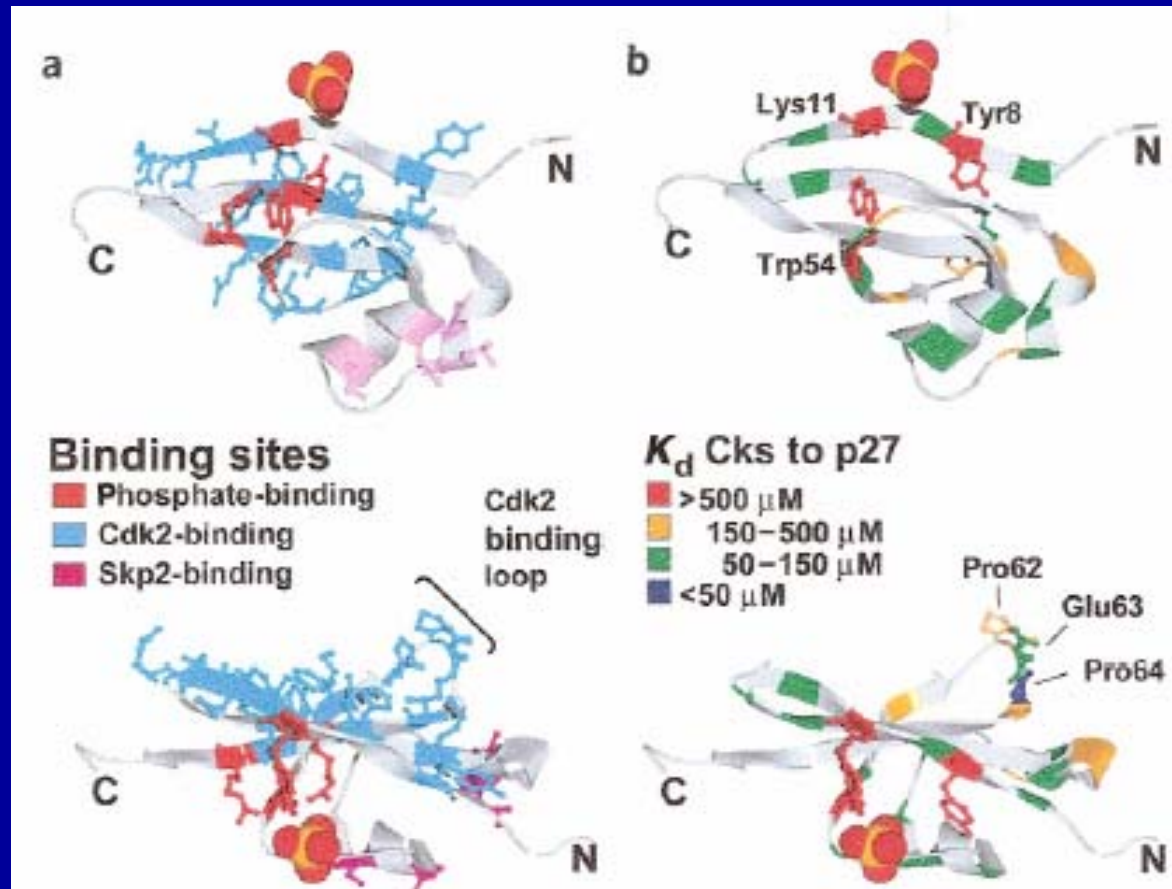
## Case-study 2

- Use fluorescein-p27 and fluorescence anisotropy to show weak binding to Cks1:
  - $K_{\text{diss}} = 70 \mu\text{M}$
- ITC: Cks1 binds much more strongly to Skp1:Skp2 than to Cdk2
  - $K_{\text{diss}} = 0.015 \mu\text{M}$
- Formation of Cks1-Skp1-Skp2 complex increases affinity of Cks1 for p27 phosphopeptide (Cdk inhibitor)  $> \times 100$ :
  - $K_{\text{diss}} = 0.47 \mu\text{M}$
- Increased 3-fold further if Cdk2 added to complex:
  - $K_{\text{diss}} = 0.15 \mu\text{M}$

# Filling out the gaps with thermodynamic cycles



# Mutational analysis highlights cross-talk between components



*Communication between distant sites important for allostery and signal transduction*