Thermodynamic measurements

When studying protein structure and function, it is essential to measure various physical quantities, including:

- **thermal and chemical stability**
- **binding affinity to a ligand**
- solubility
- dynamics and flexibility
- reactivity

One would like to predict thermodynamic properties from structure alone

When two interacting molecules come together in water

- solvent molecules are released from the binding surface
- solute molecules form bonds (H-bonds, van der Waals contact, etc)
- e.g. predict and tabulate all enthalpic and entropic changes

Stability

One of the common protein engineering goals is to stabilize a protein while maintaining its function

> increased stability means longer shelf life, obviates special treatments such as refrigeration or free-drying, makes it possible to disseminate protein drugs cheaply and effectively

> ease of maintenance and handling (no lyophilized human growth hormone that needs to be reconstituted before use)

> may require chemical modifications or site-directed mutagenesis

High solubility and resistance to (proteolytic) degradation are important

Structural integrity can be monitored by spectroscopic (CD, fluorescence), hydrodynamic and chromatographic methods

Thermal and chemical stability

Measurement of thermal/chemical stability is commonly used during protein engineering to monitor the effects of mutations and quality of design

Caveat: proteins with poorly defined core can have extremely high stability

Bryson et al, Protein Sci 7, 1404 (1998)
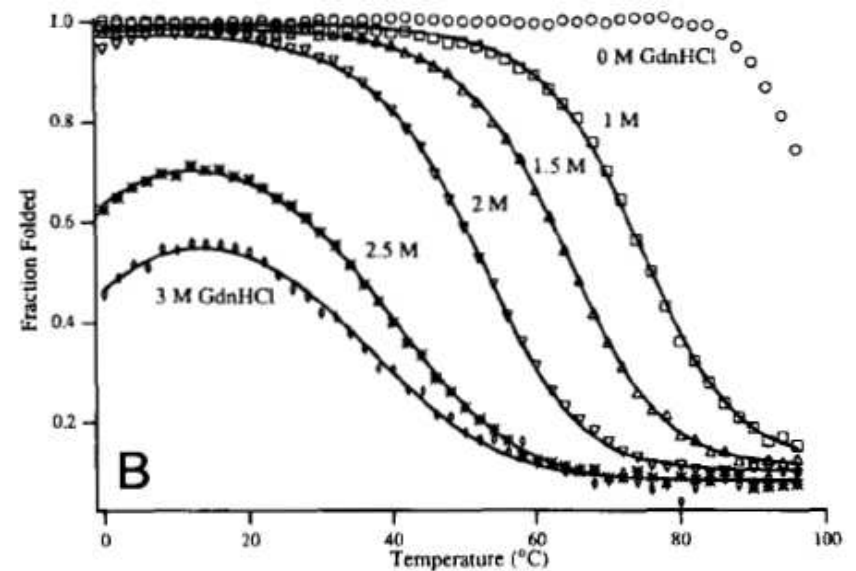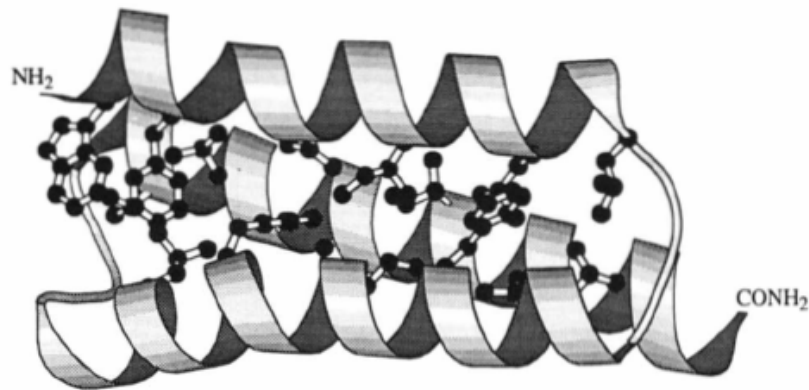
Chemical denaturation

Addition of denaturing reagents such as **urea** and **guanidine hydrochloride** (GdnHCl) can shift the equilibrium between folded and unfolded states

folded (native) $\xrightleftharpoons{K_{eq}}$ unfolded (denatured)
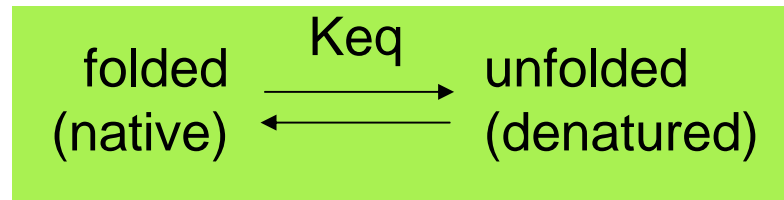
$$\Delta G = -RT \log(K_{eq})$$

Both disrupt the hydrogen bond network within the protein as well as the protein-solvent interaction, thus destabilizing the folded structure

- – mechanism of denaturation is not fully understood
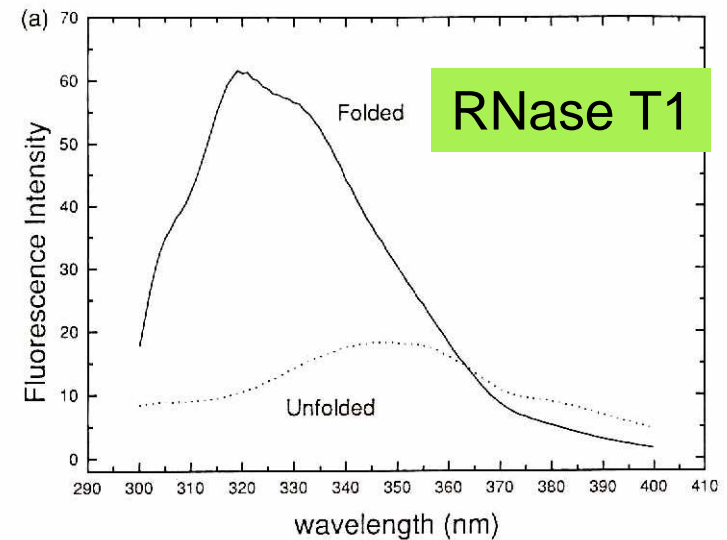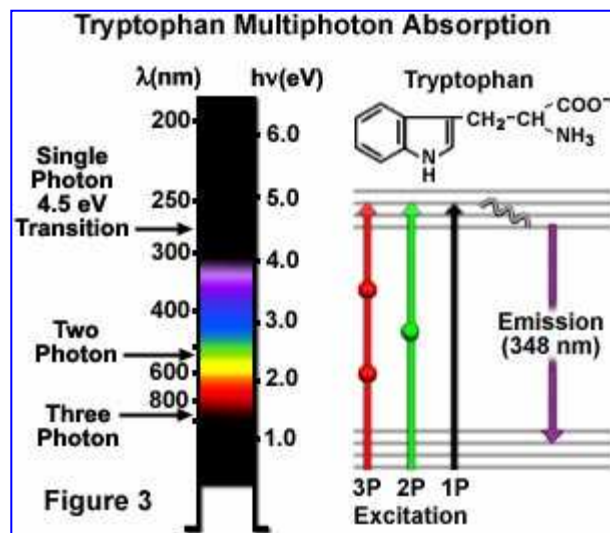- – see Bennion and Daggett, PNAS 100, 5142 (2003)

In part due to its net charge, GdnHCl is about twice as effective as urea in inducing protein denaturation (i.e. will unfold protein at roughly half the concentration)
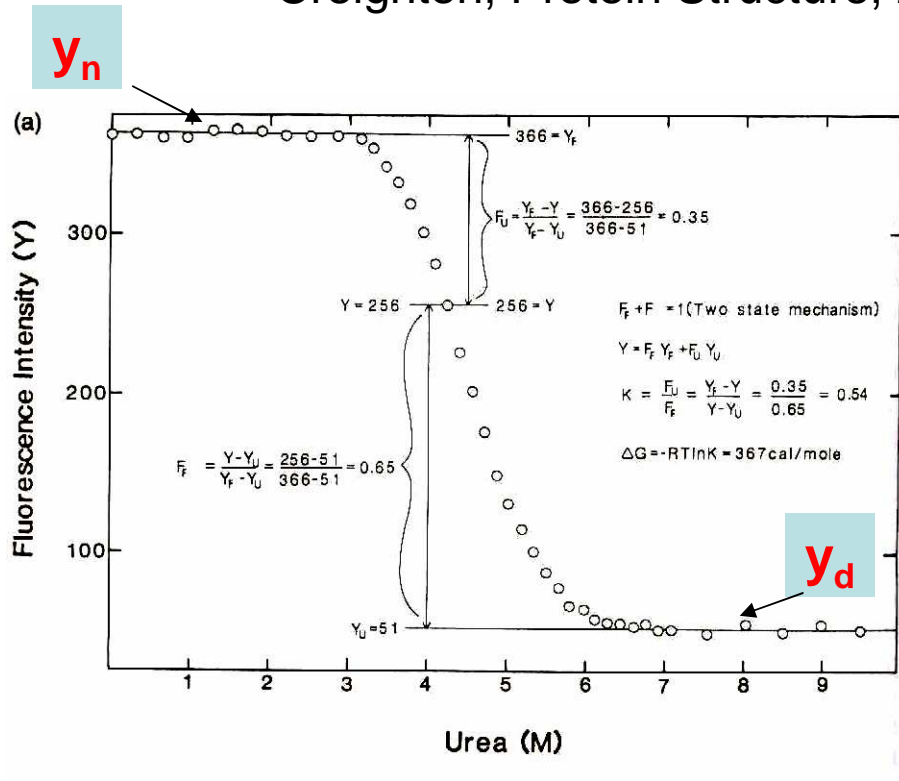
Tryptophan fluorescence

Fluorescence is absorption of light at wavelength λ_a and subsequent emission of light at λ_e ($\lambda_e > \lambda_a$)

Trptophan fluorescence ($\lambda_a \sim 278$ and $\lambda_e \sim 348$ nm) is used to study protein conformation (i.e. tertiary structure)

The exact λ_e and intensity of fluorescence depends critically on the local environment around the indole ring

RNase T1

http://www.molecularexpressions.com

y$_n$

(a)

Fluorescence Intensity (Y)

300

200

100

$366 = Y_F$

$F_U = \dfrac{Y_F - Y}{Y_F - Y_U} = \dfrac{366 - 256}{366 - 51} = 0.35$

$Y = 256 \quad 256 = Y$

$F_F + F = 1 \text{(Two state mechanism)}$

$Y = F_F Y_F + F_U Y_U$

$K = \dfrac{F_U}{F_F} = \dfrac{Y_F - Y}{Y - Y_U} = \dfrac{0.35}{0.65} = 0.54$

$\Delta G = -RT \ln K = 367 \, cal/mole$

$F_F = \dfrac{Y - Y_U}{Y_F - Y_U} = \dfrac{256 - 51}{366 - 51} = 0.65$

y$_d$

$Y_U = 51$

1 2 3 4 5 6 7 8 9

Urea (M)

$$K_{eq} = \frac{f_d}{f_n}$$

$$f_d = \frac{y_n - y}{y_n - y_d}$$
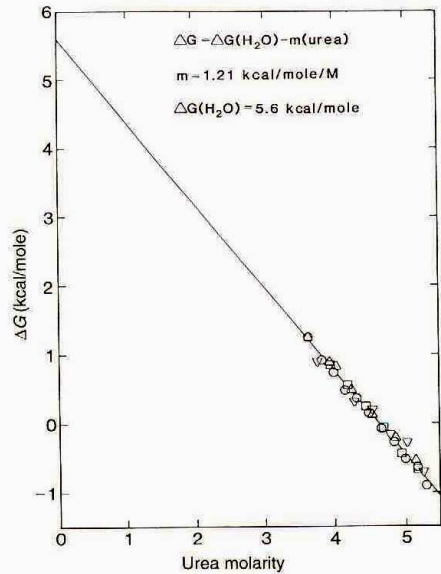
$$f_n = 1 - f_d = \frac{y - y_d}{y_n - y_d}$$

$$-RT \log K_{eq} = -RT \log\left(\frac{y_n - y}{y - y_d}\right) = \Delta G$$

Require sufficient data points in all three regions:
  – pre, post and transition

Need to ensure equilibrium has been reached
  – may take a long time (> hrs)
  – slowest during the transition
  – reversing the direction of the experiment should yield an identical curve
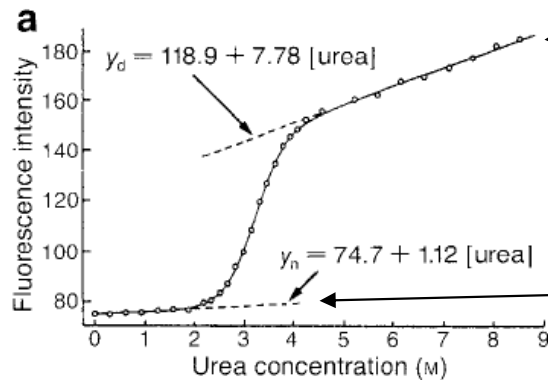
Measuring Keq is feasible only within the range of 0.1 – 10

$$\Delta G = \Delta G(H_2O) - m[D]$$

where [D] is the concentration of denaturant, e.g. urea, GdnHCl, and m measures the dependence of ΔG on [D]

At the midpoint of the denaturation curve, $[D]_{1/2} = \Delta G(H_2O)/m$

$$y_d + m_d[D]$$

$$y_n + m_n[D]$$

$$
\begin{array}{l}
y_n, m_n \\
\Delta G(H_2O), m \\
y_d, m_d
\end{array}
$$

$$y = \frac{(y_n + m_n[D]) + (y_d + m_d[D]) * \exp\{-(\Delta G(H_2O) - m[D])/RT\}}{1 + \exp\{-(\Delta G(H_2O) - m[D])/RT\}}$$

Thermal denaturation

As for chemical denaturation, determine$_{CD, 220 \, nm}$
Keq for the transition region

$$\Delta G = -RT \log(K_{eq}) = aT + b$$

$$\Delta G_{T_m} = 0$$
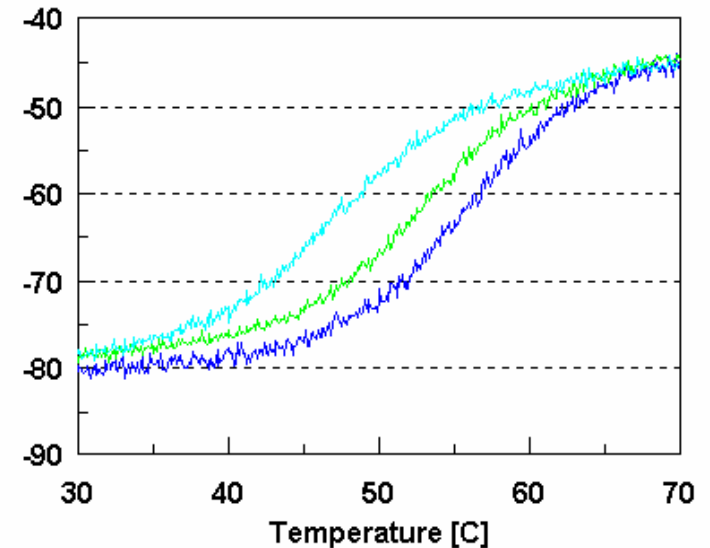
$$T_m = -\frac{b}{a}$$

$$\Delta G_{T_m} = 0 = \Delta H_{T_m} - T\Delta S_{T_m}$$

$$\Delta H_{T_m} = T\Delta S_{T_m} = T \frac{d\left(\Delta G\right)}{dT} = T \bullet a$$

or

$$\log(K_{eq}) = \frac{\Delta H}{RT} - \frac{\Delta S}{R} \qquad \text{(van't Hoff)}$$

$$\frac{d\left(\log(K_{eq})\right)}{d\left(1/T\right)} = \frac{\Delta H_{vh}}{R} \quad \boxed{\text{from a linear fit}}$$

In general,

$$\frac{d(\Delta H)}{d(T)} \neq 0$$

$$\Delta\left(\frac{dH}{dT}\right) = \Delta C_p = C_p(U) - C_p(F)$$

$$\Delta G(T) = \Delta H_{T_m}\left(1 - \frac{T}{T_m}\right) - \Delta C_p\left(T_m - T + T\log\frac{T}{T_m}\right)$$
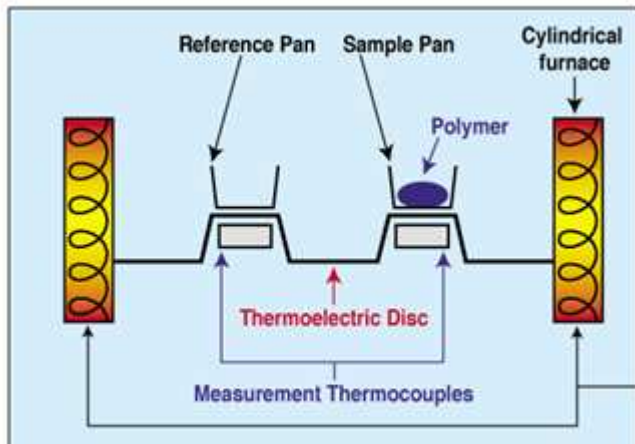
Differential scanning calorimetry (DSC)

DSC can measure heat capacity of a sample by computing the amount of heat required to raise the temperature by a fixed amount:
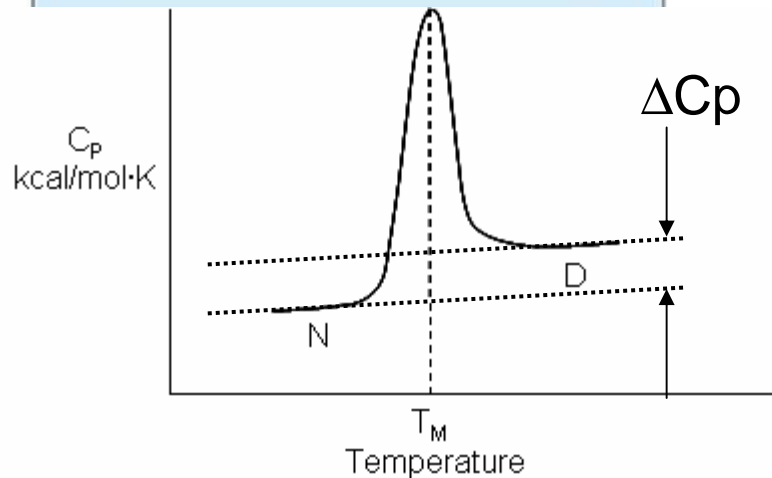
$$C_p = \frac{\Delta Q}{\Delta T}$$

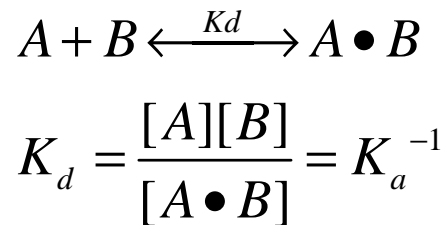When the protein denatures, its heat capacity increases, resulting in a positive change in Cp

Alternatively, if a calorimeter is not available, ΔCp can be estimated:
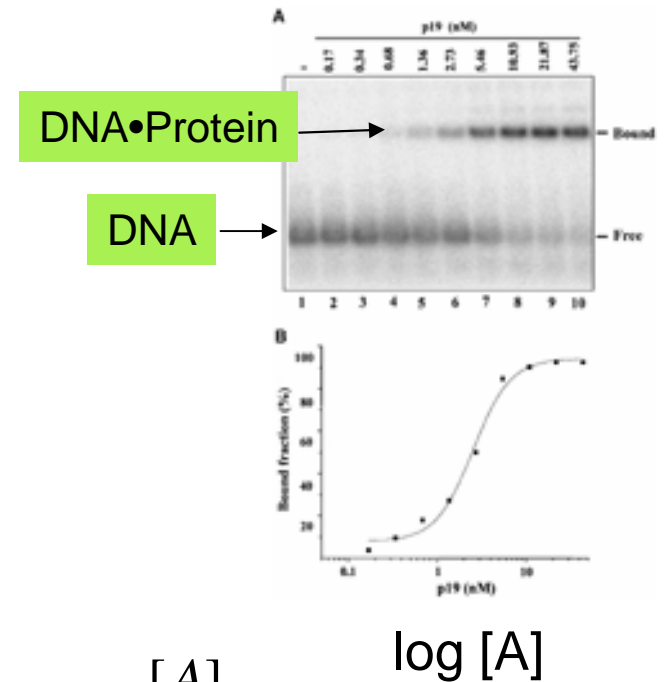
$$C_p \approx 172 + 17.6N - 164 * (\# \text{ of SS})$$

Binding affinity

Characterizing binding affinity is essential for many biochemical processes

$$A + B \xleftrightarrow{\quad Kd \quad} A \bullet B$$

$$K_d = \frac{[A][B]}{[A \bullet B]} = K_a^{-1}$$

DNA•Protein

DNA

log [A]

If [A] >> [B], then the fraction of B bound to A is $f = \dfrac{[A]}{[A] + K_d}$

Kd is the concentration of A where half of B is bound
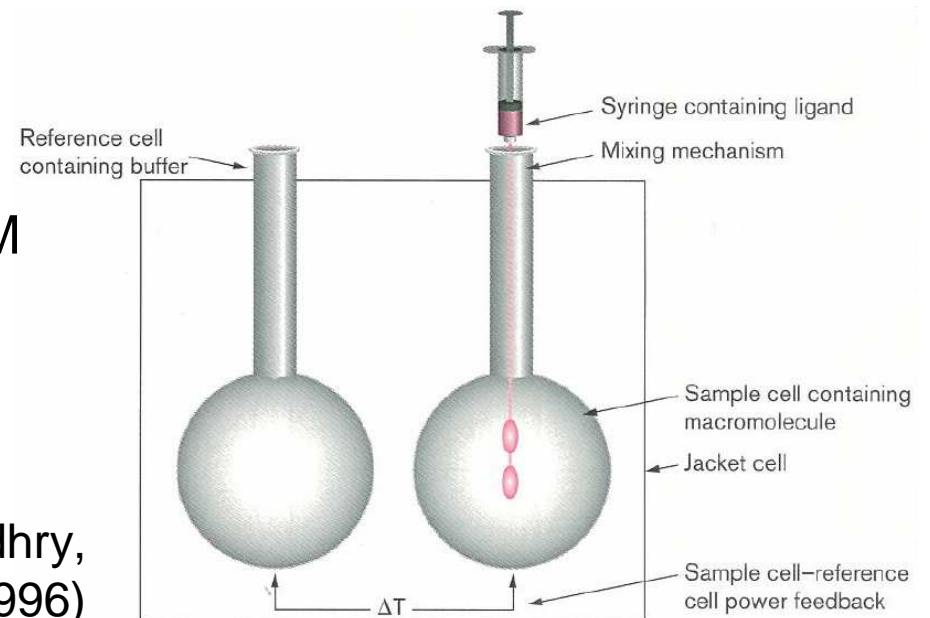
Isothermal titration calorimetry (ITC)

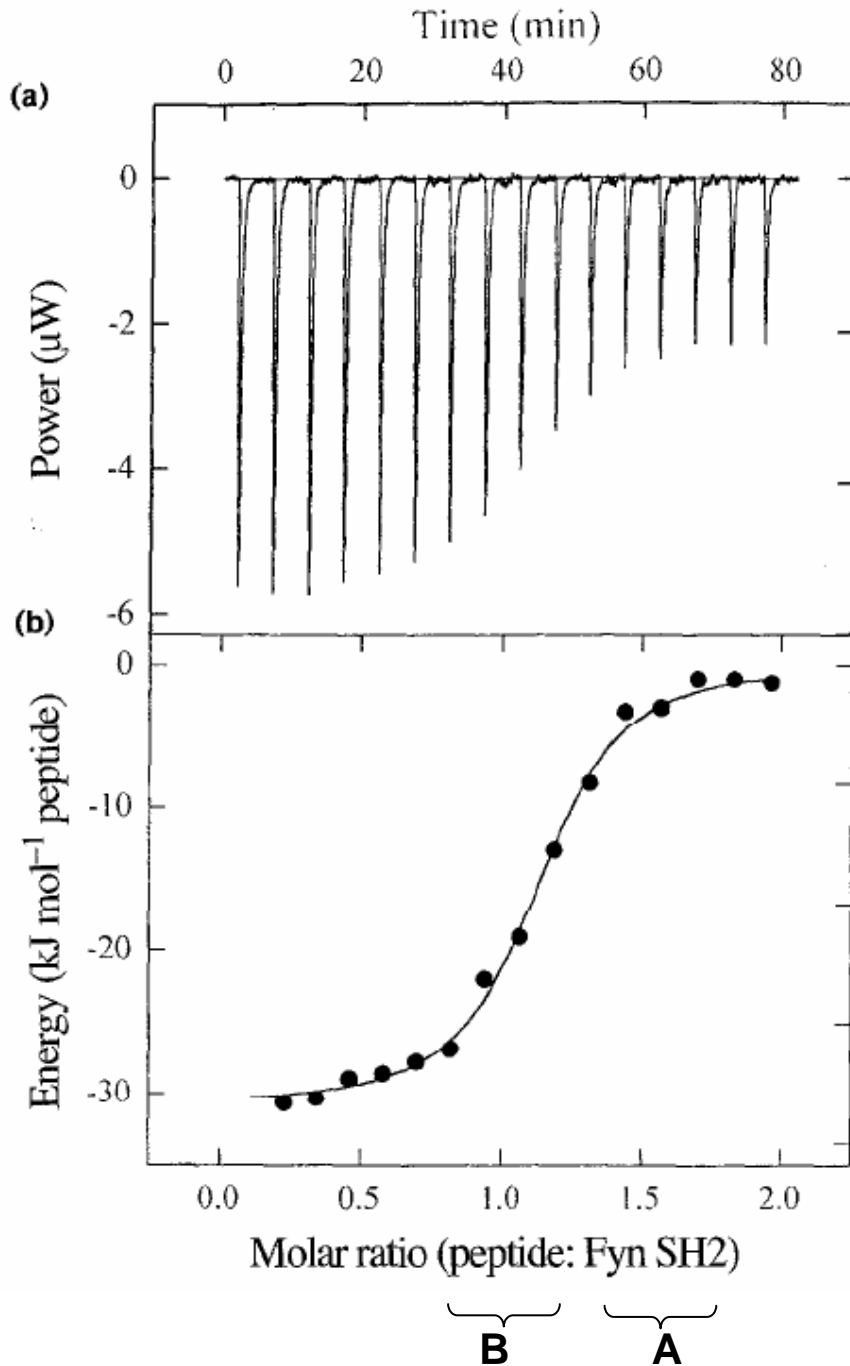ITC can measure most of commonly used thermodynamic parameters, including ΔH, ΔS, ΔC_p

Electronics monitors the amount of heat that was added to keep the two cells at the same temperature ($\sim \mu W$)

When a second component is added to the sample cell, either more or less heat is required to maintain the sample cell at the same temperature, depending on whether the binding
is endothermic or exothermic

Can measure Kd between 1 nM – 1mM

Ladbury and Chowdhry,
Chem & Biology 3, 791 (1996)

Reference cell containing buffer

Syringe containing ligand

Mixing mechanism

Sample cell containing macromolecule

Jacket cell

Sample cell–reference cell power feedback

ΔT

(a)

Time (min)

Power (µW)

(b)

Energy (kJ mol^{-1} peptide)

Molar ratio (peptide: Fyn SH2)

B   A

For the reaction A+nB ➜ A•nB,

the heat input/output Q is related to the enthalpy of binding (ΔH), association constant (K_a) and stoichiometry of binding (n) as
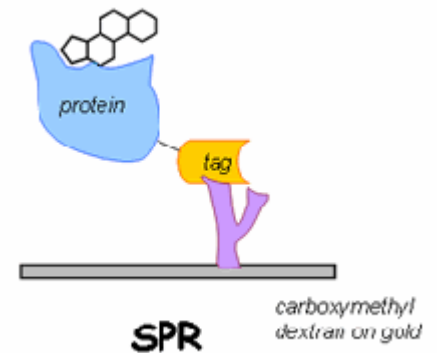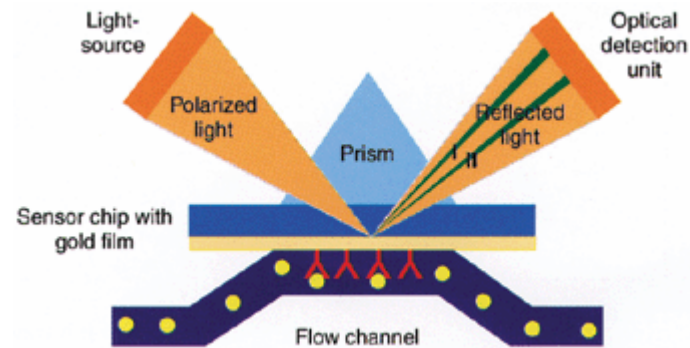
$$Q = \frac{1}{2} n A V \Delta H \left\{ X - \left[X^2 - \frac{4B}{nA} \right]^{\frac{1}{2}} \right\}$$

$$X = 1 + \frac{B}{nA} + \frac{1}{nK_a A}$$

A is the total amount of sample #1 in the cell, **B** is the final amount of sample #2 added, **V** is the volume of the liquid

Surface plasmon resonance (Biacore)

SPR measures the binding of nanoparticles (e.g. protein, DNA, small molecules, carbohydrates, etc) on a metal surface (silver or gold) by monitoring the change in the local index of refraction


When polarized light undergoes total internal reflection, the intensity of the reflected light is reduced at a specific incident angle due to the resonance energy transfer between evanescent wave and surface plasmons
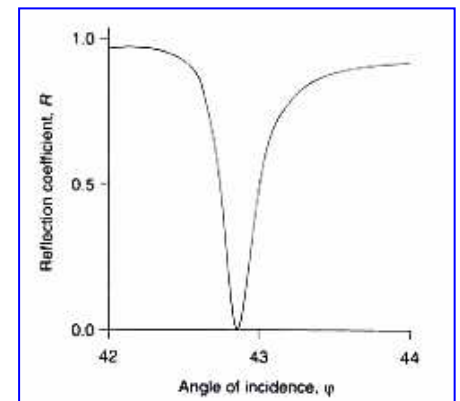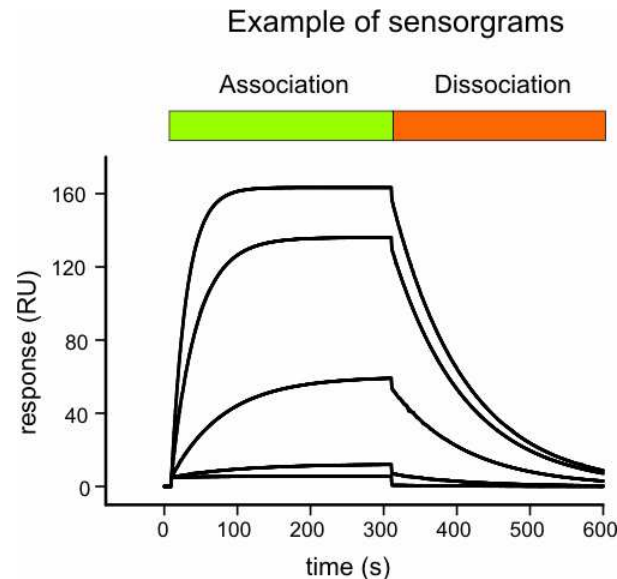
Refractive index varies with the molecular weight of the bound substrate

binding of a ligand to the receptor immobilized on the surface changes the molecular weight of the complex, hence the angle of minimum intensity

Kinetic information may be obtained from SPR by measuring Kon and Koff

$$A + B \xrightleftharpoons[Koff]{Kon} A \bullet B$$

$$K_d = \frac{K_{off}}{K_{on}}$$

Example of sensorgrams
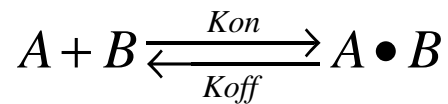
Reference: Silin and Plant, TIBiotech 15, 353 (1997)