Isothermal Titration Calorimetry (ITC) for the Evaluation of Macromolecule-Ligand Interactions

$$[M] + [L] \Leftrightarrow [ML]$$

Some methods for obtaining thermodynamic parameters for biomolecules:

1) van't Hoff analysis

2) Calorimetry

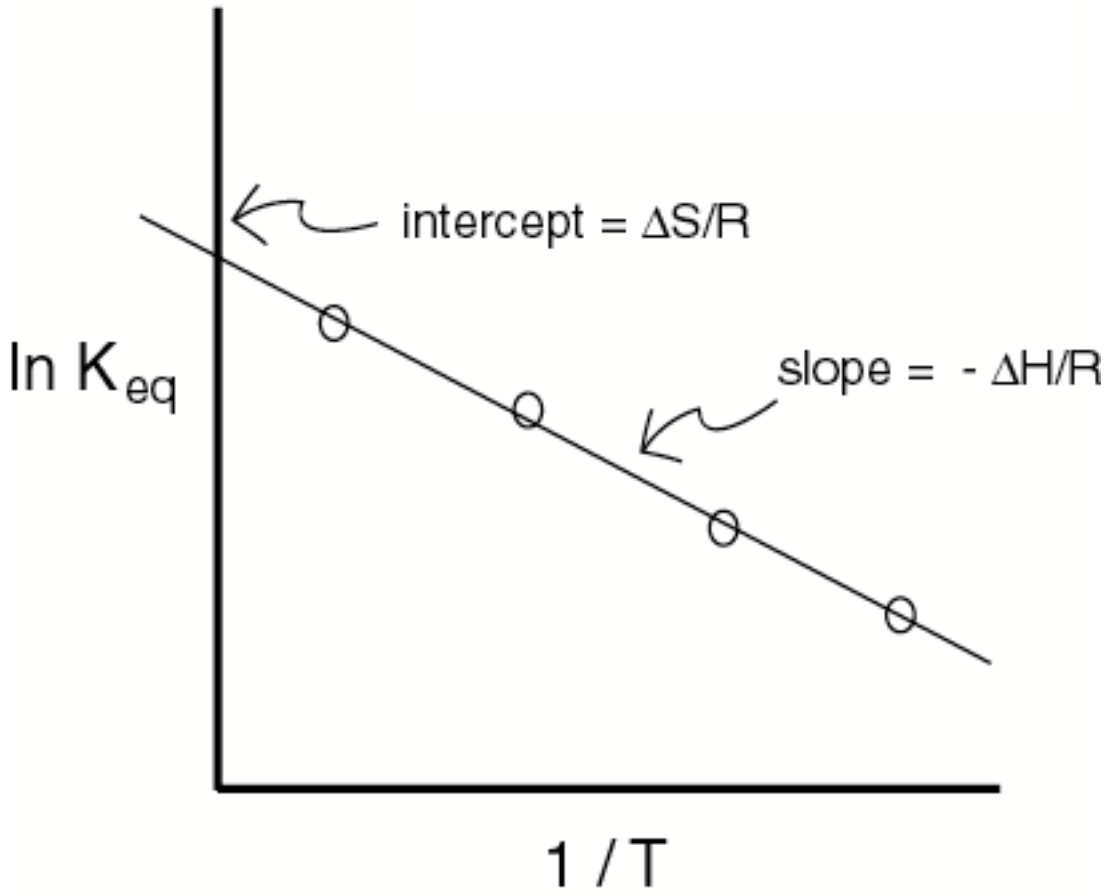
van’t Hoff analysis can be performed any time you can measure equilibrium constant as a function of temperature.

$$\Delta G = - RT \ln K_{eq} \quad \text{and} \quad \Delta G = \Delta H - T\Delta S$$

Therefore: $- RT \ln K_{eq} = \Delta H - T\Delta S$

Rearrange: $\ln K_{eq} = - (\Delta H/RT) + (\Delta S/R)$

$$y = m\,x + b$$



$$\ln K_{eq} = -(\Delta H/RT) + (\Delta S/R)$$

What's nice about van't Hoff analysis?

If you can measure [M], [L], [ML] by any spectroscopic method, as a function of temperature, you can obtain thermodynamic parameters.

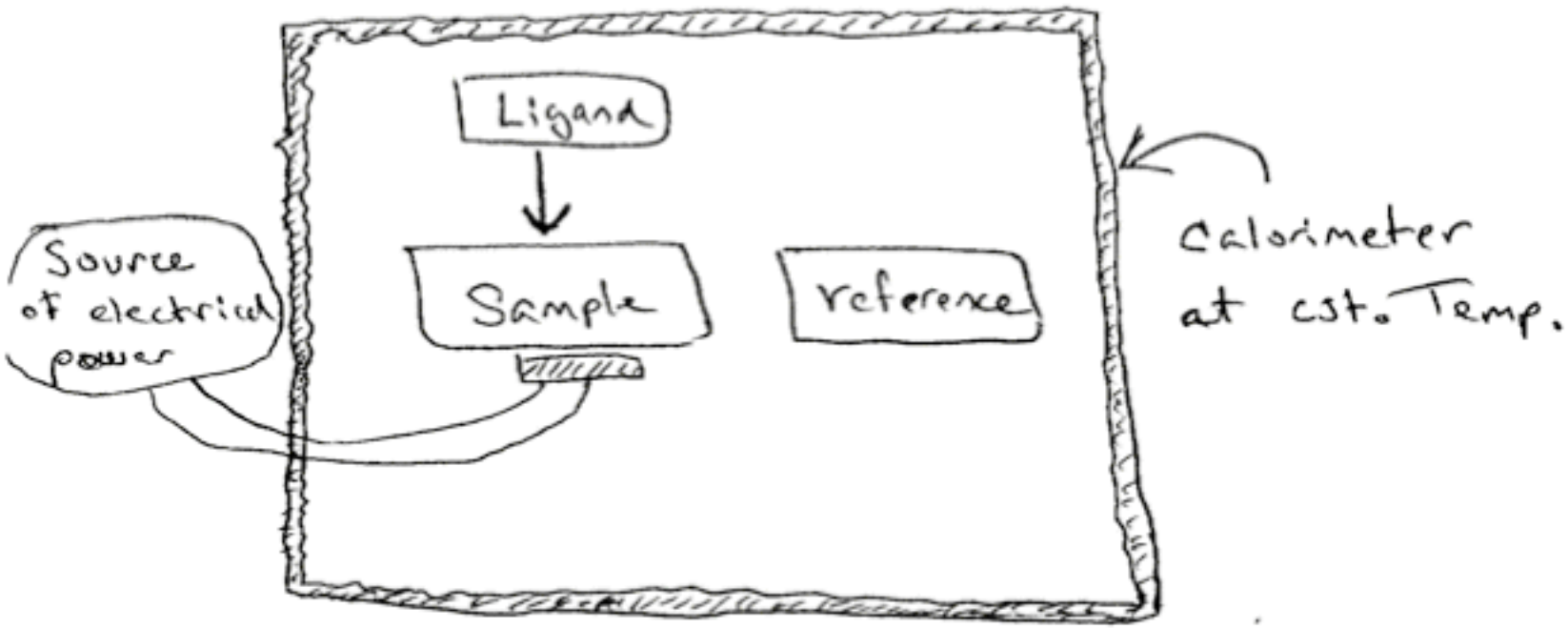
Or, any time that you can measure Keq at different temperatures, you can obtain thermodynamic parameters.

Why is van't Hoff analysis flawed?

- van't Hoff analysis assumes ΔH & ΔS & sample's heat capacity do not vary with temperature (this is not exactly correct).
- usually only a small temperature range is accessible when working with biochemical systems.

Isothermal Titration Calorimetry (ITC) provides a more rigorous alternative to van't Hoff analysis.

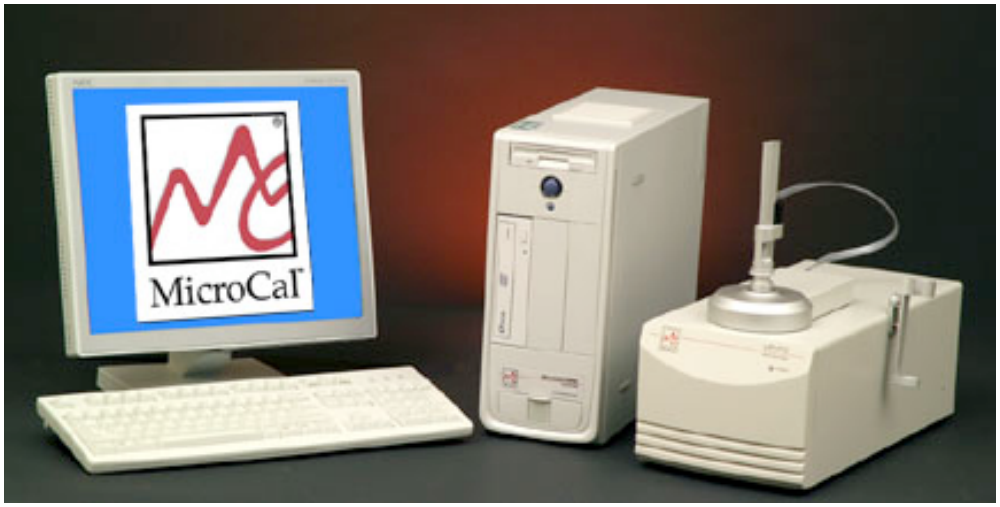
Experimental arrangement:



* Ligand is added to sample a few microliters at a time, at ≈ 1 minute intervals.

* Electrical energy is used to keep sample and reference at same temperature.

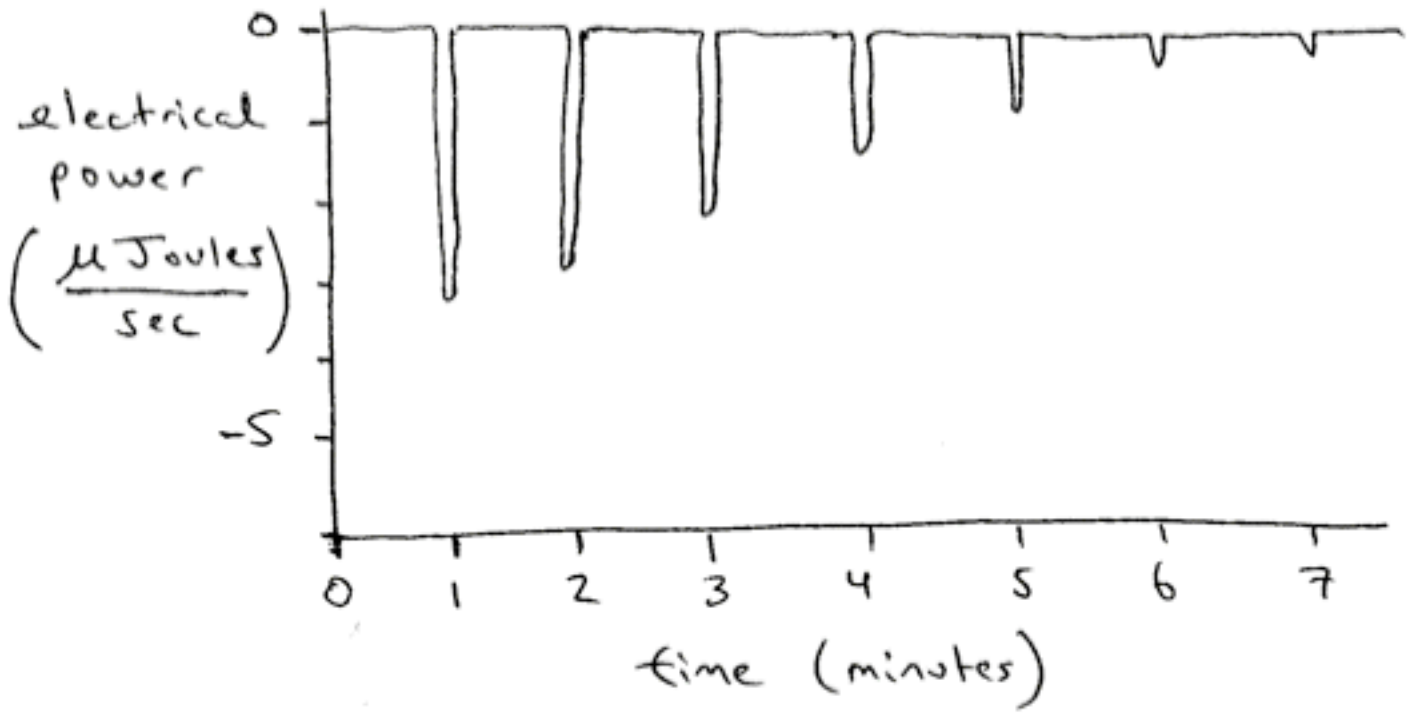
ITC instruments (Microcal)



The iTC_{200} utilizes a 200 µl cell versus the 1.4 ml cell in previous models.

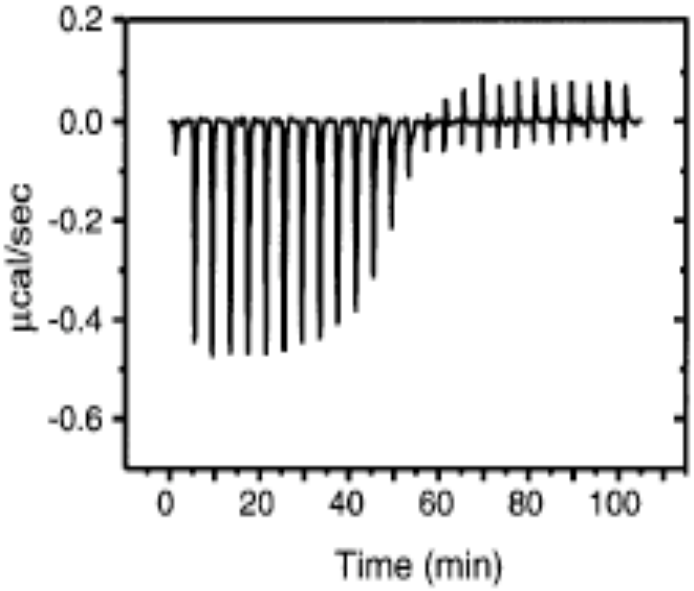
ITC sample cells,
0.2 ml and 1.4 ml.

What is recorded:



* An aliquot of ligand solution is added to the sample once per minute.
* This releases heat.
* Electrical energy is required to re-equilibrate the sample and reference temperatures.

Some typical ITC data - mixing of two proteins that bind, with the release of heat:



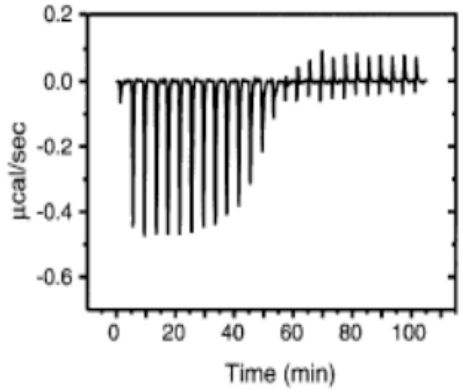
As ligand is added, binding sites on the macromolecule become saturated (this results in progressively less electrical energy being required to re-equilibrate the sample and reference cells).

Information regarding Keq for ligand binding is contained within the ITC data, in addition to information regarding ΔH.

Data analysis:

$\Delta q(i)$ = enthalpy change upon each mixing event *i*.
$\Delta q(i)$ is obtained by integrating each peak in ITC trace.

$\Delta q(i)$ is a function of n, [M], [L], V, ΔH, K_{eq}



where:

n	=	# binding sites
[M]	=	conc. Macromolecule
[L]	=	conc. Ligand
V	=	sample volume
ΔH	=	enthalpy change upon ligand binding
K_{eq}	=	equilibrium cst for ligand binding

total [M], total [L], V are usually known.

n, ΔH, Keq are found by minimizing the difference between the observed and calculated peak areas in the ITC trace.

ΔG can be calculated from Keq
ΔS can be calculated using ΔH & ΔG

Solve for n, ΔH, Keq.

These are found by minimizing the difference between the observed and calculated peak areas in the ITC trace.

$$\Delta q_i = n [M]_{total} V_{cell} \cdot \Delta H \cdot R$$

where $R \equiv$ root of quadratic equation:

$$\gamma_i^2 - \gamma_i \left(1 + \frac{1}{n K_{eq} [M]_{total}} + \frac{[L_i]_{total}}{n[M]_{total}} \right) + n [L_i]_{total} [M]_{total} = 0$$

and $$\gamma_i = \frac{[L_i]_{bound}}{[M]_{total}}$$

or just remember:

$$\Delta q_i = \text{function of } n, [M], [L], V, \Delta H, K_{eq}$$

An experimental consideration:

[M] and [L] must be chosen so that there is a significant amount of both free and bound ligand present during the titration.

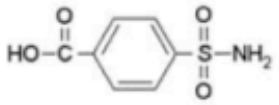
- for very tight binding ($K_d \approx 10^{-9}$ M) the ITC experiment must be performed at very low ligand concentrations. This results in only very small power pulses needed to keep temperature constant, which results in significant experimental error.

Another experimental consideration:

Only a very small amount of heat is released in each ligand binding event, so a large amount of sample must be used so there is enough heat released to be detectable.

(as a consequence, typically 1 to 10 mg amounts of macromolecule are needed for an ITC titration)

Example of an application of ITC:

Carbonic anhydrase (29 kDa enzyme) +  <=> Carbonic anhydrase : CBS bound complex

Carboxysulfonamide (CBS)

$$[M] + [L] \iff [ML]$$

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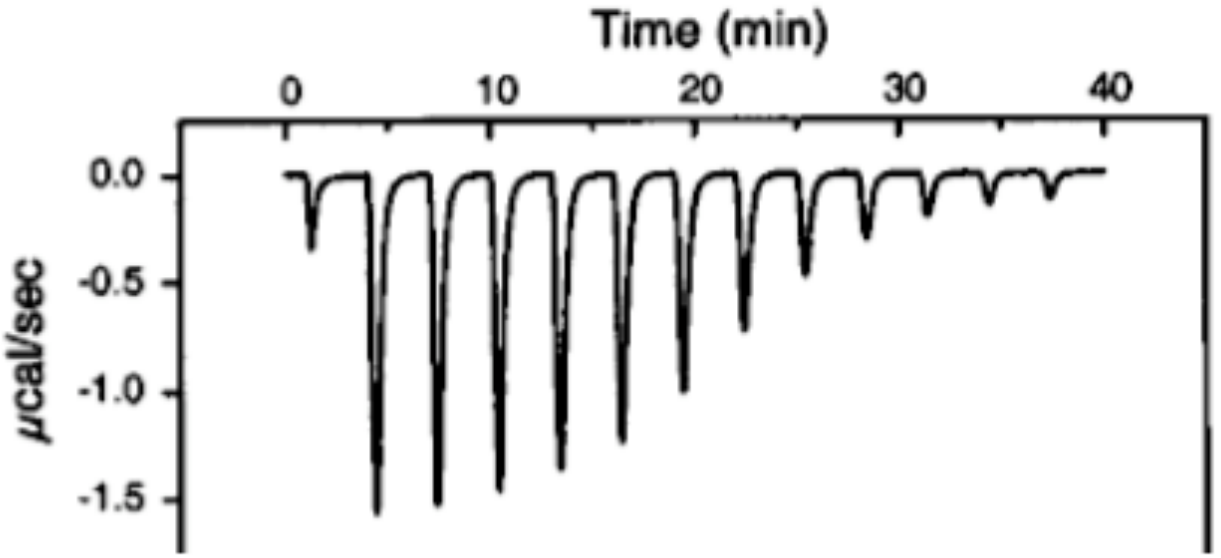
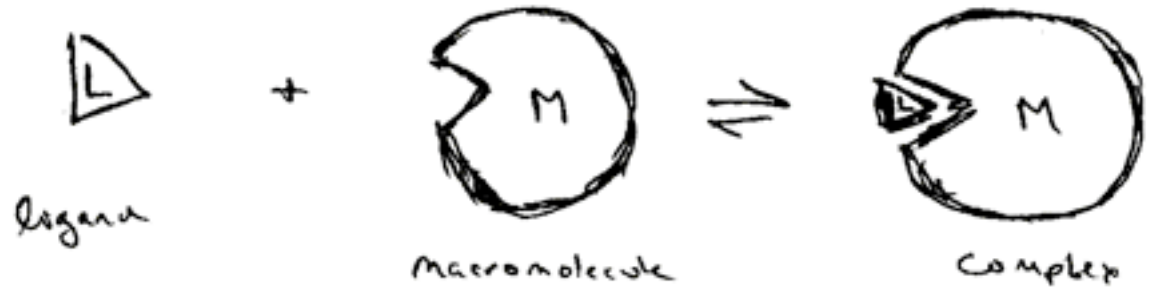
ITC data: CBS binding to carbonic anhydrase.



K_D (nM)	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$\Delta S°$ [cal/(mol K)]
760 ± 30	−8.3 ± 0.3	−11.6 ± 0.4	−11 ± 1



$\Delta G < 0$ (for binding reactions, when equilibrium is on the side of the complex)

ΔS usually negative (surface of M becomes more ordered upon ligand binding)

$\therefore$ ΔH must be substantially negative, to overcome the unfavorable entropy change upon ligand binding.

(Remembering $\Delta G = \Delta H - T\Delta S$)

Summary: Isothermal Titration Calorimetry (ITC)

Good things about ITC:

Accurate determination of binding (K_d) and thermodynamic (ΔG, ΔH, ΔS) parameters for ligand-macromolecule interactions.

ITC does not make the approximations that are included in a van't Hoff type of analysis.

Not such good things about ITC:

Large (perhaps 10 mg) quantities of material are required, in order to detect the small amount of heat released upon mixing macromolecule and ligand.

In comparison, gel-mobility shift assays can be carried out with << 1 mg of material.

Surface Plasmon Resonance (SPR)

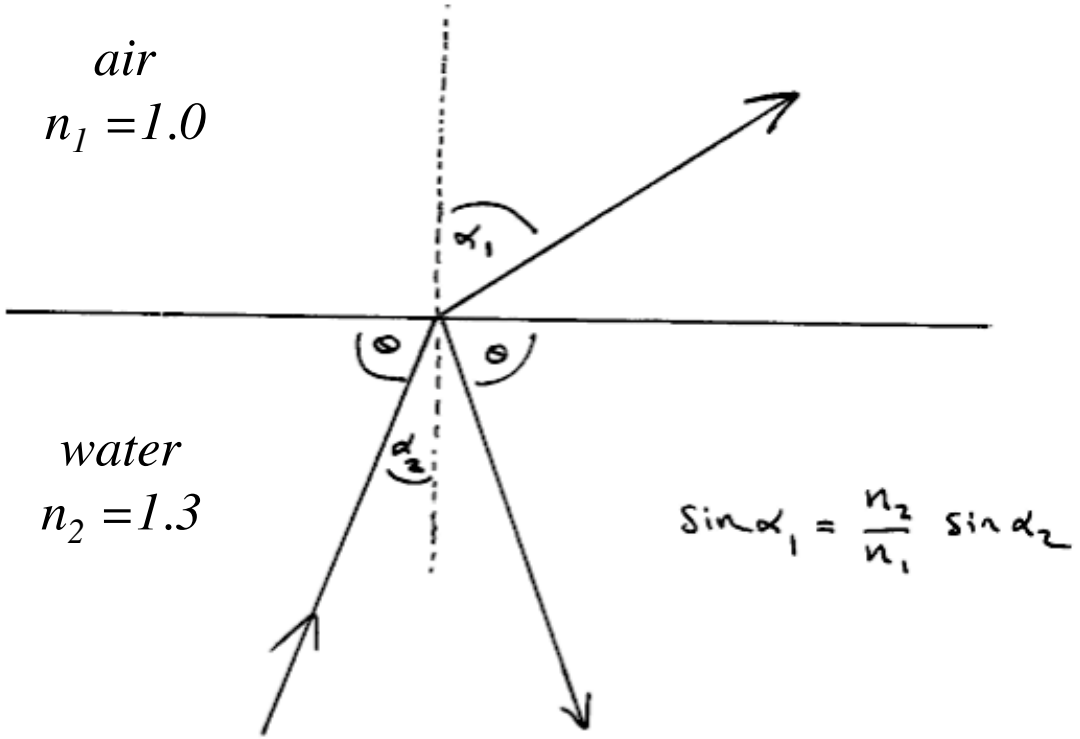
also known as

Biomolecular Interaction Analysis (BIA)

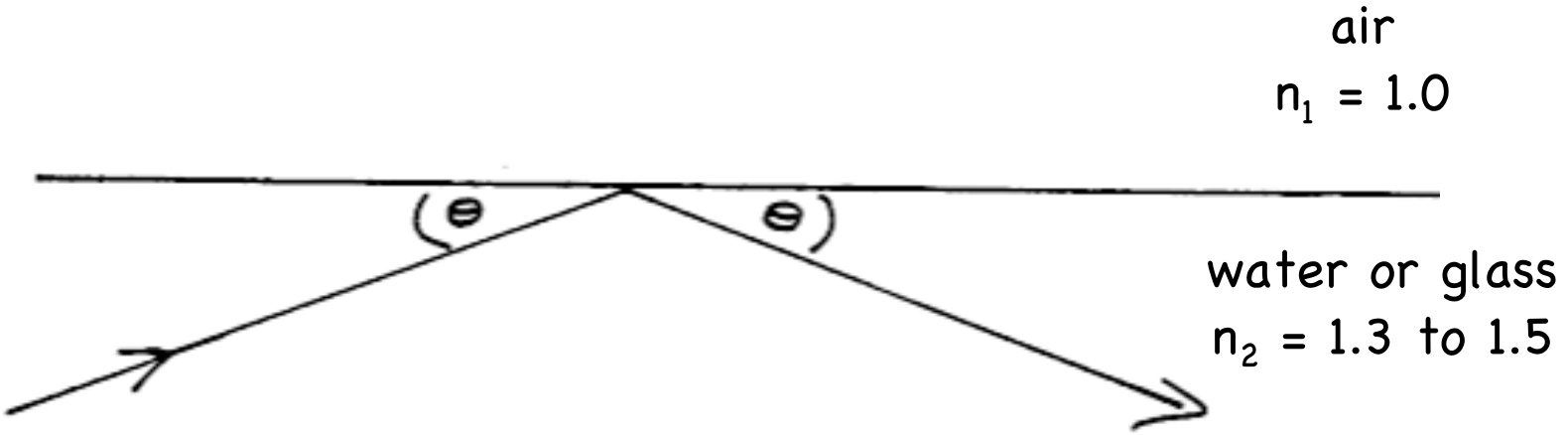
SPR is a relatively new method for detecting and characterizing interactions between biomolecules.

What is SPR?

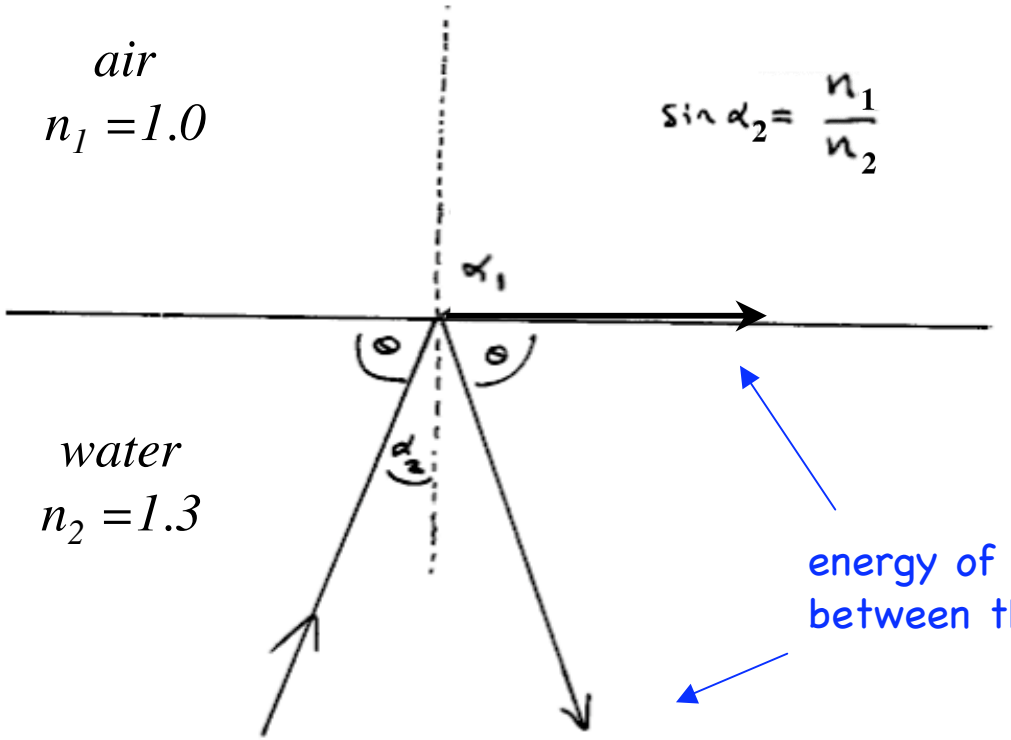
First, recall what happens to light at the interface of 2 transparent media with different index of refraction:



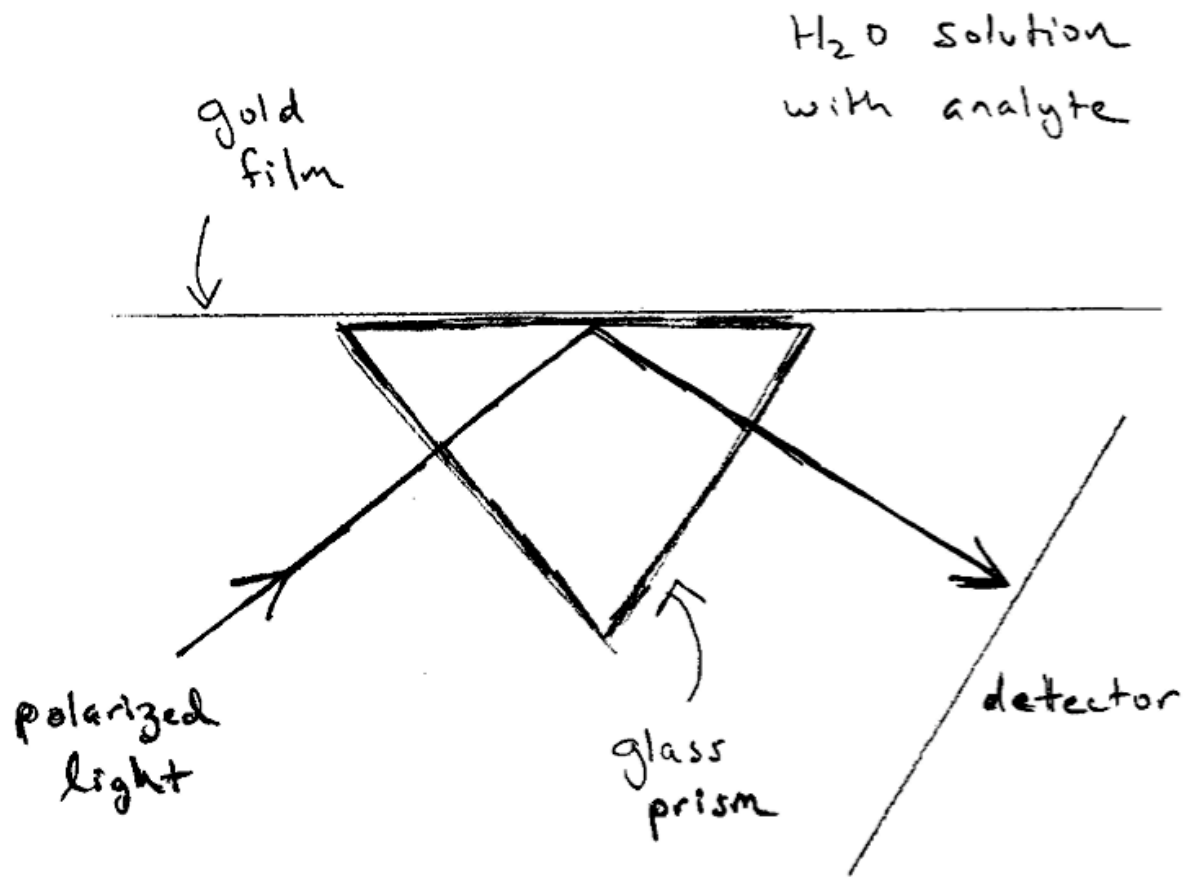
At low angle of incidence, there is "total internal reflection".



With some angle of incidence, the refracted ray can be directed parallel to the interface:



Apparatus for SPR experiment:



Detector measures the intensity of the reflected light.

Surface Plasmon Resonance (SPR)

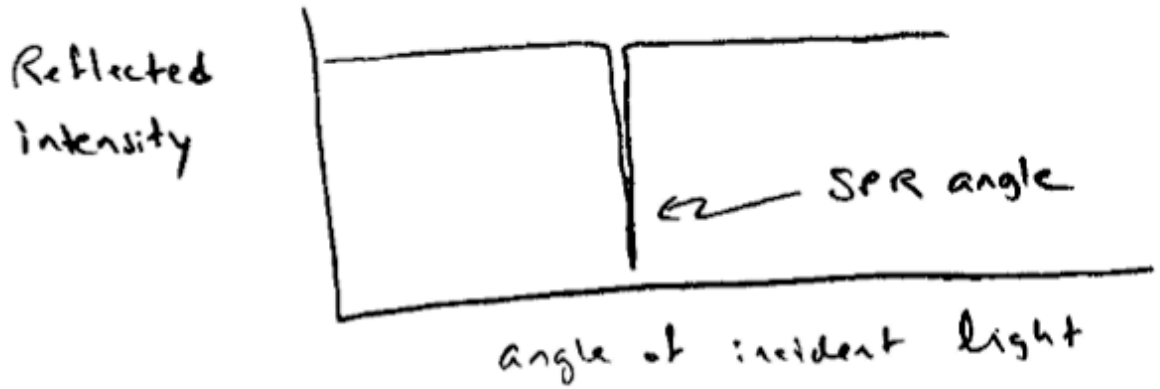
At just the right incident angle (the SPR angle), light energy can be transferred to electrons in a gold film between the glass and the solution, exciting a group of electrons in the gold into a state called a "plasmon".

Any change in refractive index at the gold surface changes the incident angle required for the light to create a plasmon in the metal.

Creating the plasmon causes a decrease in the intensity of the reflected light (since the energy for creating the plasmon comes from the light).

The incident angle at which SPR occurs depends on the refractive index of the material on each side of the metal.

What is measured?

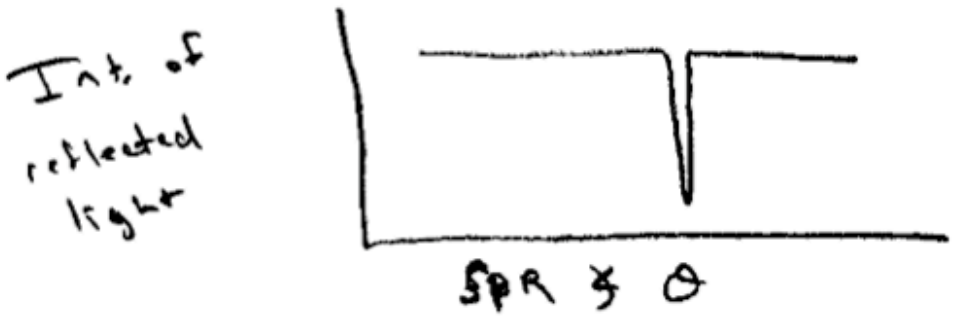


At the SPR angle, the energy of the incident photons is efficiently transferred to the plasmon electrons.

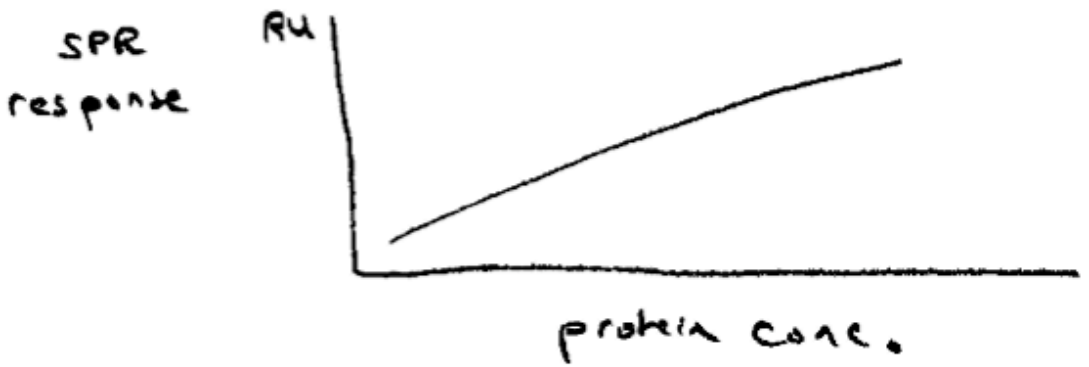
Accumulation of protein at the surface of the gold film causes the index of refraction of the solution to change.

This causes a change in the angle required for SPR to occur.

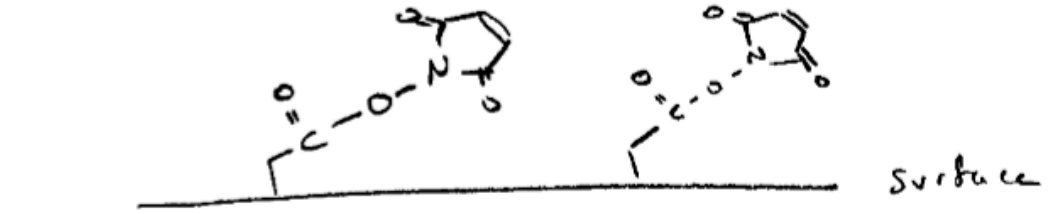
The SPR angle depends on protein concentration at the interface of the 2 regions with different refractive index :



"SPR response" = a measure of the change in SPR %.



For SPR to be useful in measuring protein-protein (or protein-ligand) interactions, it is necessary to attach the target molecule to the surface at the interface between the 2 regions that differ in refractive index.

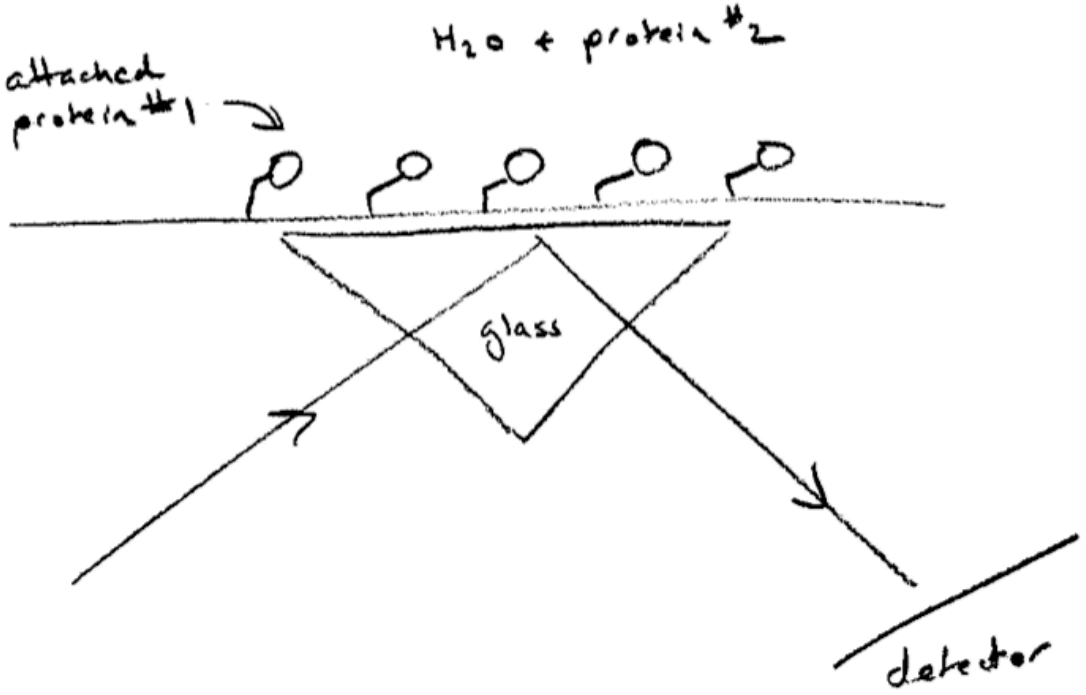


reacts with amines & aldehydes.

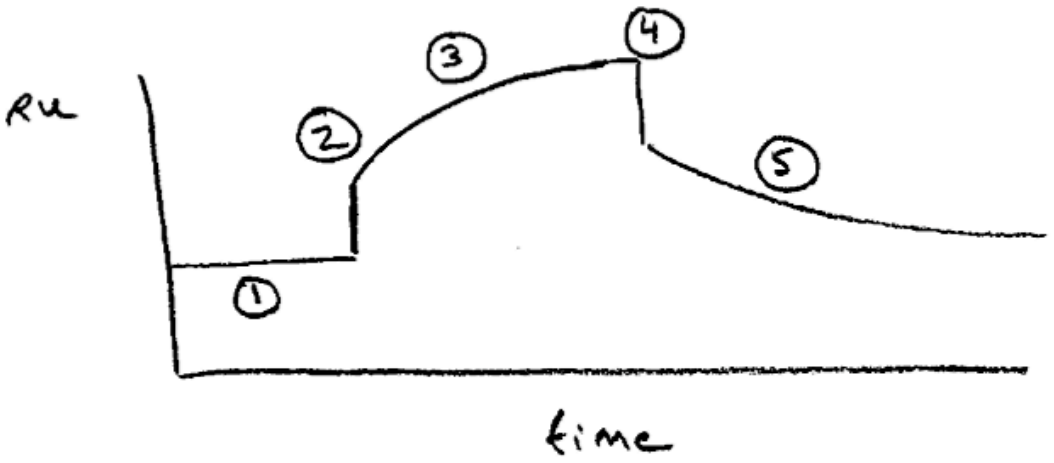
Proteins can be attached to the surface by reaction with lysine amine groups.

Also, Histidine-tagged proteins can be attached to the surface using their ability to bind to metals with 2+ charge:

SPR – ready to detect binding interaction between protein #1 & protein #2 :



The SPR experiment:



① analyte region contains buffer only. Protein #1 is attached to surface

② Introduce protein #2 into solution.

③ binding kinetics ⇒ kon

④ wash away protein #2

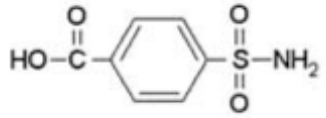
⑤ dissociation kinetics ⇒ koff

$$K_d = {}^{k_{off}}/_{k_{on}}$$

SPR study of CBS binding to carbonic anhydrase.

Carbonic anhydrase
(29 kDa enzyme)

+



Carboxysulfonamide
(CBS)

<=>

Carbonic anhydrase : CBS
bound complex

Direct comparison of binding equilibrium, thermodynamic, and rate constants determined by surface- and solution-based biophysical methods

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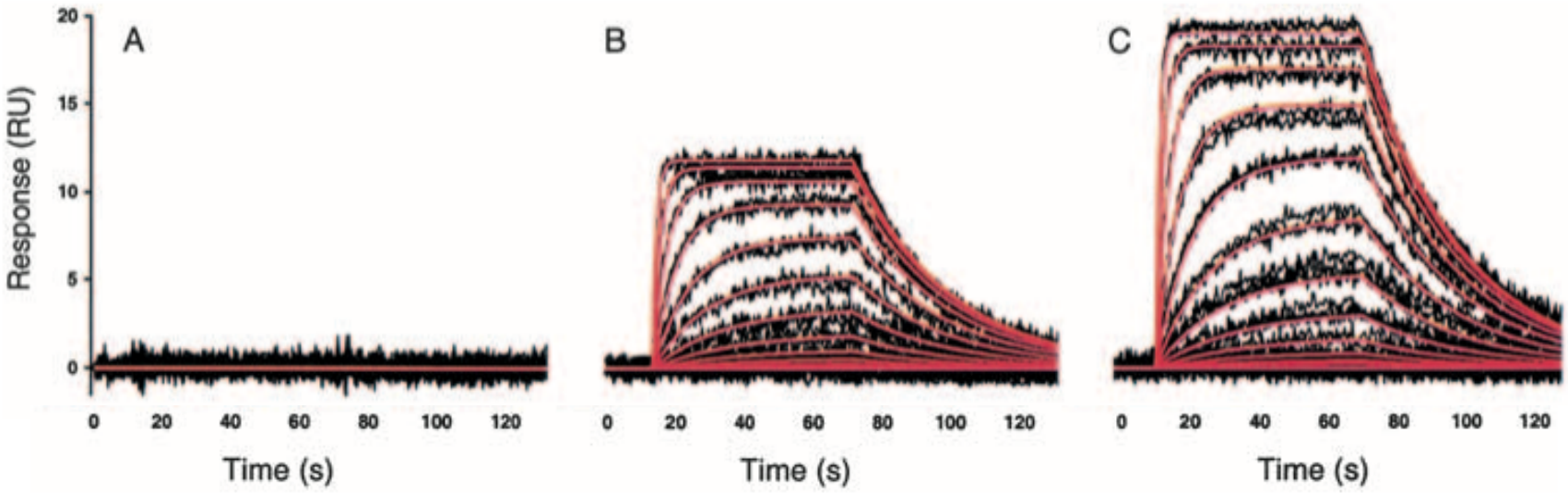
SPR data: CBS binding to carbonic anhydrase.

No carbonic anhydrase on SPR chip:

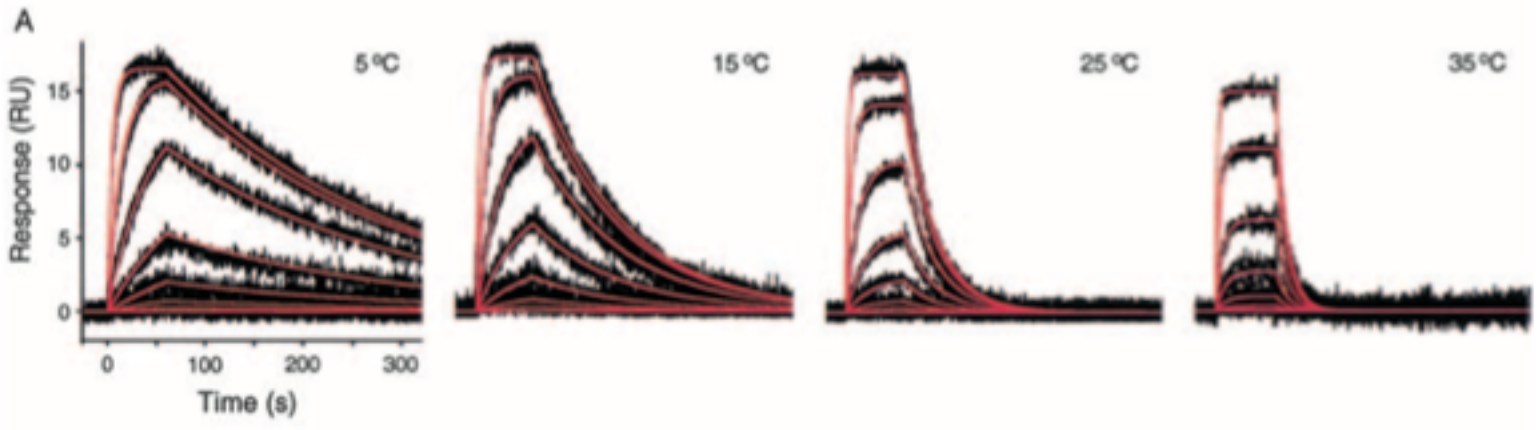
Carbonic anhydrase bound to SPR chip:

Higher concentration of carbonic anhydrase bound to SPR chip:



k_{on} and k_{off} are used to find K_d.

SPR data can be used to estimate ΔH for CBS binding to carbonic anhydrase, using a van't Hoff approach.



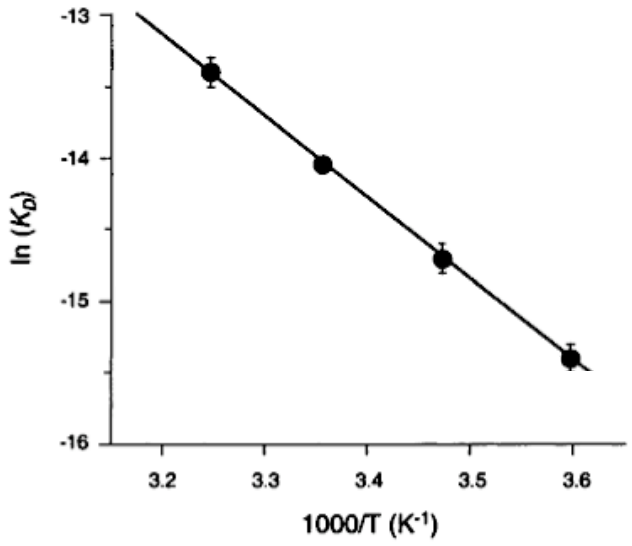


Table 1. *Kinetic and thermodynamic constants determined for CA II/sulfonamide interactions using SPR, ITC, and SFF*

Analysis method	Sulfonamide compound	T (°C)	Exp[a]	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)	ΔG° (kcal/mol)	ΔH° (kcal/mol)	ΔS° [cal/(mol K)]
SPR	CBS	25	6	$(4.8 \pm 0.2) \times 10^4$	0.0365 ± 0.0006	760 ± 30	-8.3 ± 0.3	-11.6 ± 0.4	-11 ± 1
ITC	CBS	25	5	—	—	730 ± 20	-8.4 ± 0.2	-11.9 ± 0.4	-12 ± 1