Binding equilibria - kinetics and thermodynamics

One-step, one-site binding

$$P + L \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} PL$$

P: free protein; L: free ligand; PL: protein-ligand complex

k_{off}: unimolecular rate constant, inverse of τ_B: mean lifetime of complex

k_{on}: bimolecular rate constant, measures the probability of a productive encounter between protein and ligand; for diffusion-controlled reactions is usually taken equal to $10^7 – 10^9$ M^{-1} s^{-1}

$$K_d = \frac{[P][L]}{[PL]} = \frac{k_{off}}{k_{on}}$$

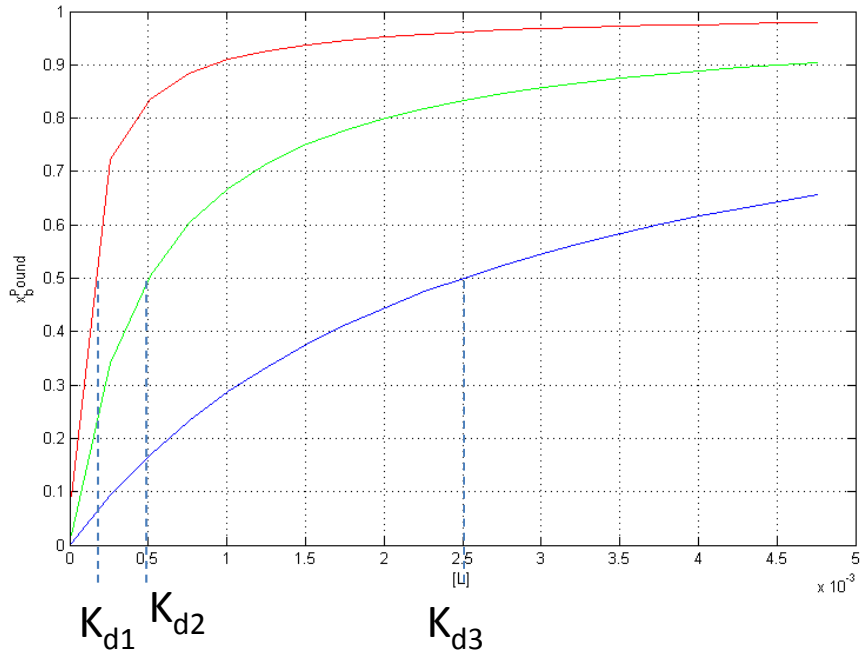
law of mass action

K_d: dissociation constant, expressed in M units, approximates real thermodynamic constant which is referred to activities rather than concentrations and is unitless; depends on temperature

Binding equilibria – population fractions

$$x_b^P = \frac{[PL]}{[P]+[PL]} = \frac{[L]}{[L]+K_d}$$

hyperbolic equation
Langmuir binding isotherm



Increasing [L] increases x^P_b although by progressively smaller amounts
$[L] << K_d$, x^P_b is proportional to [L]
$[L] = K_d$, the protein is half-saturated
$[L] >> K_d$, the protein is completely saturated
Ligands of weaker affinity have larger K_d and require more ligand to saturate the binding site

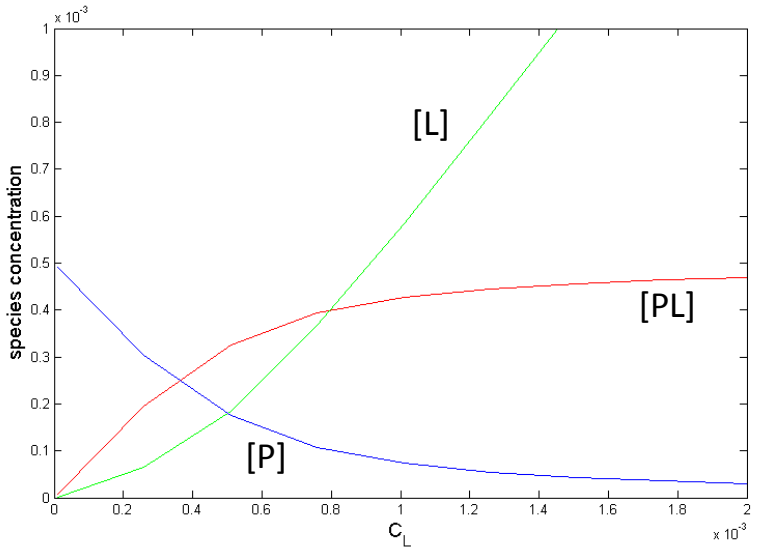
Binding equilibria – population fractions

[L] is usually not an accessible quantity in NMR practice

We need to refer to the total protein and total ligand concentrations instead, for this we use the mass balance expressions and solve the quadratic equation for [PL]

$$C_P = [P] + [PL] \qquad C_L = [L] + [PL]$$

$$[PL] = \frac{C_P + C_L + K_d - \sqrt{(C_P + C_L + K_d)^2 - 4C_P C_L}}{2}$$



Competitive binding equilibria

$$PI \underset{k^{I}_{off}}{\overset{k^{I}_{on}}{\rightleftharpoons}} P + I + L \underset{k^{L}_{off}}{\overset{k^{L}_{on}}{\rightleftharpoons}} PL$$

$$x^{P}_{b,+I} = \frac{[L]}{[L] + K_{d,app}}$$

$$K_{d,app} = K_{d}\left(1 + \frac{[I]}{K_{I}}\right)$$

Because $K_{d,app} \geq K_d$, $x^{P}_{b,+I} \leq x^{P}_{b}$ reflecting the reduction of available receptor sites for L

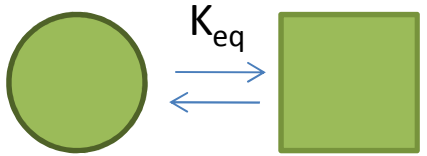
Competitive equilibria may be exploited to obtain conditions more amenable for the determination of K_d

Exchange kinetics and appearance of the NMR spectrum

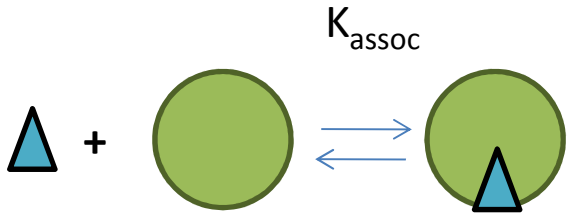
Chemical exchange refers to any process in which a nucleus exchanges between two or more environments in which its NMR parameters (e.g. chemical shift, scalar coupling, or relaxation) differ.

These may be intermolecular or intramolecular processes.

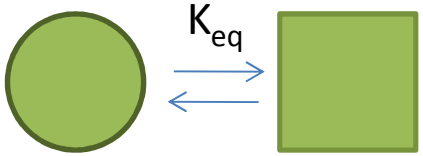
Intramolecular exchange (conformational exchange)
- motions of protein side chains
- helix-coil transitions of nucleic acids
- unfolding of proteins
- conformational equilibria
- tautomerization



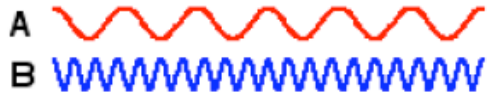
Intermolecular exchange:
- binding of ligands to macromolecules
- protonation/deprotonation equilibria of ionizable groups
- isotope exchange processes
- enzyme catalyzed reactions



Two-site exchange

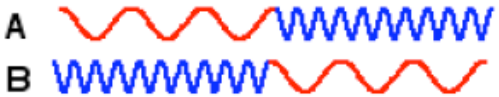


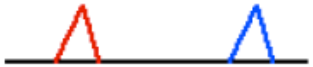
$k_{ex} = k_{on} + k_{off} = 0$



no exchange

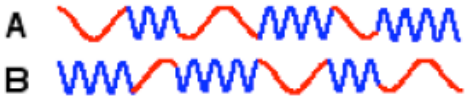
$k_{ex} << |\Omega_A - \Omega_B|$

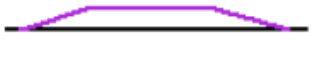




slow exchange

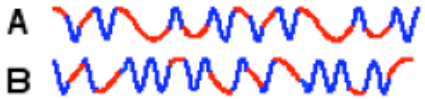
$k_{ex} \sim |\Omega_A - \Omega_B|$



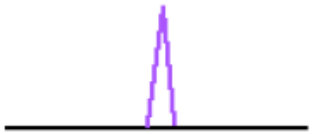


intermediate exchange

$k_{ex} \sim |\Omega_A - \Omega_B|$



$k_{ex} >> |\Omega_A - \Omega_B|$



fast exchange

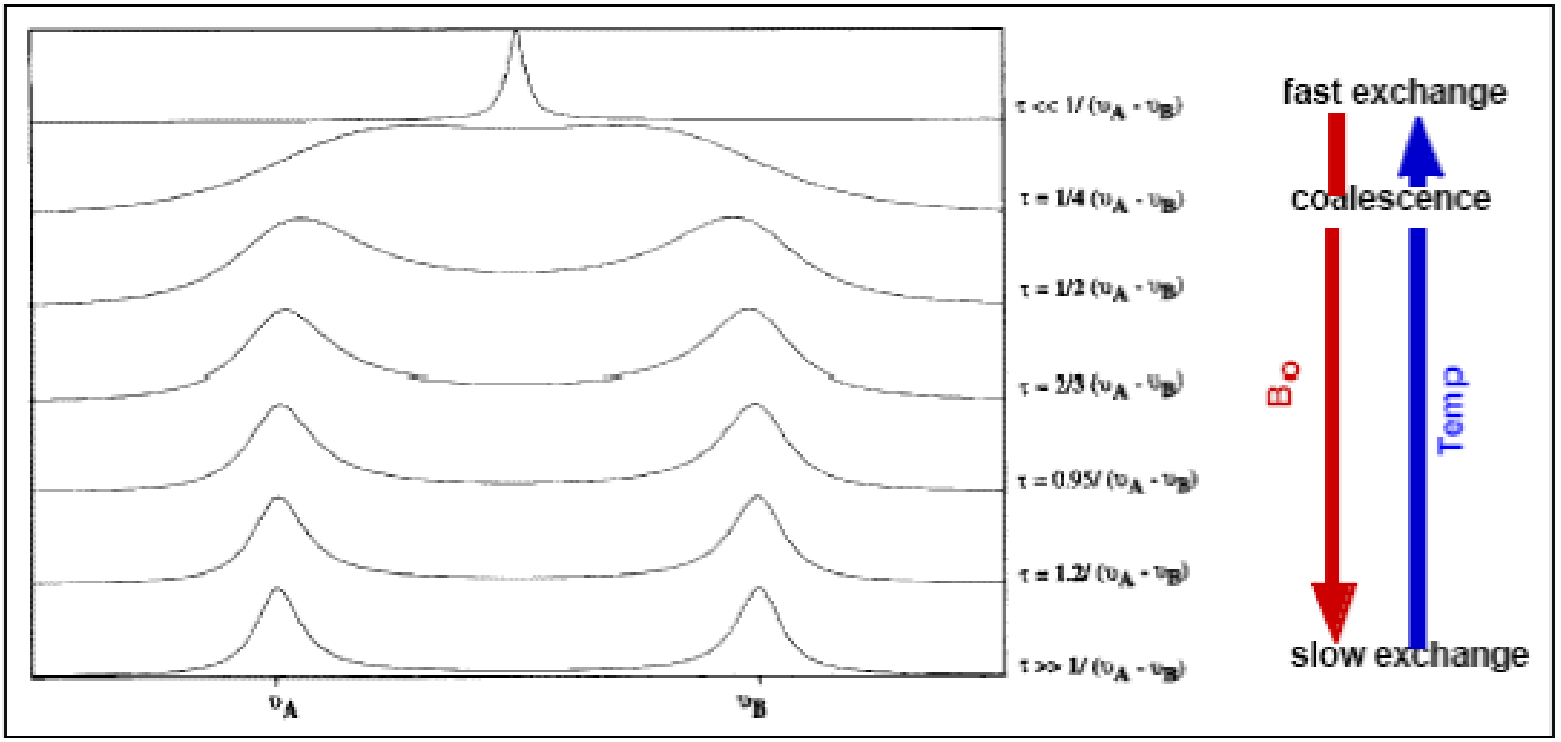
Modulation of exchange regime

Temperature dependence of line shape

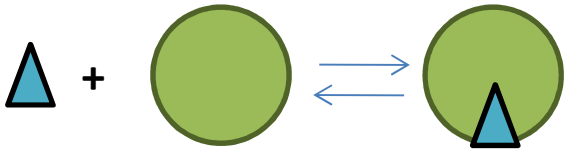
Increasing temperature shifts toward the fast exchange limit

Field dependence of line shape

Increasing field shifts toward the slow exchange limit

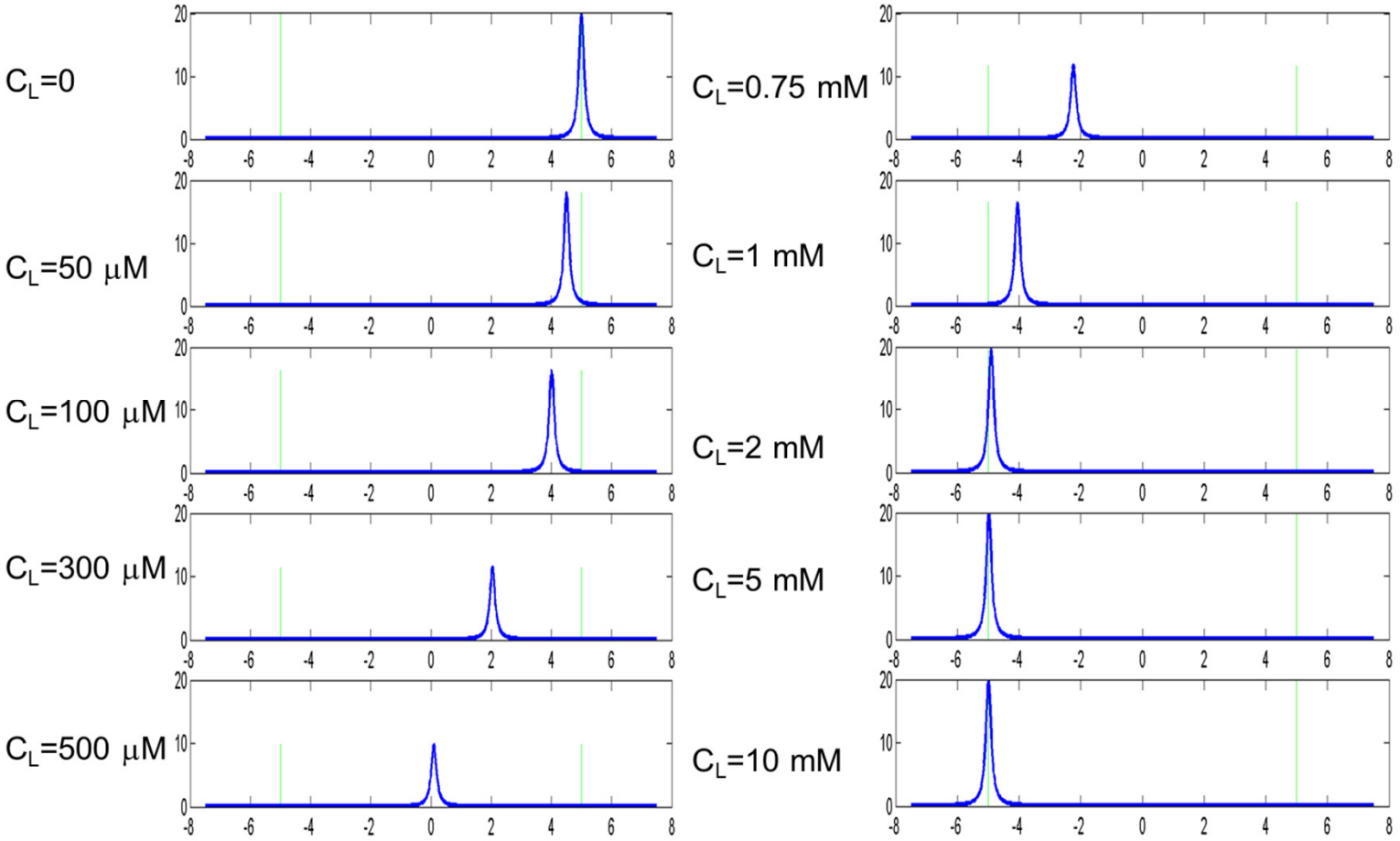


Simulation of two-state second order exchange



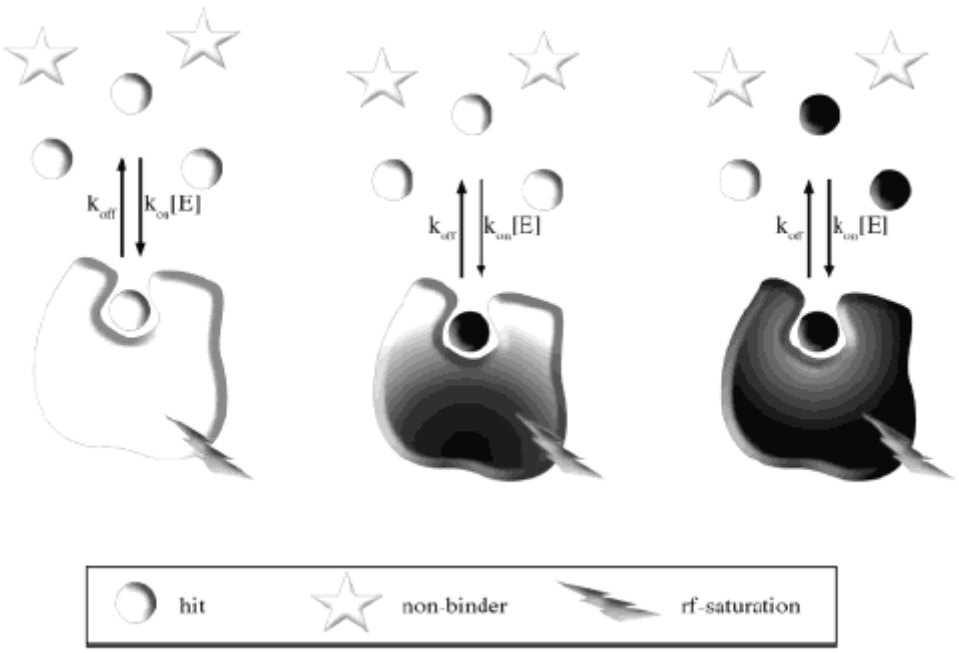
$P + L \rightleftharpoons PL$

K_d=100μM; C_P=1 mM; k_{ex}=ΔΩ*500 (fast exchange); observing protein signal



Ligand-based experiments
Saturation transfer

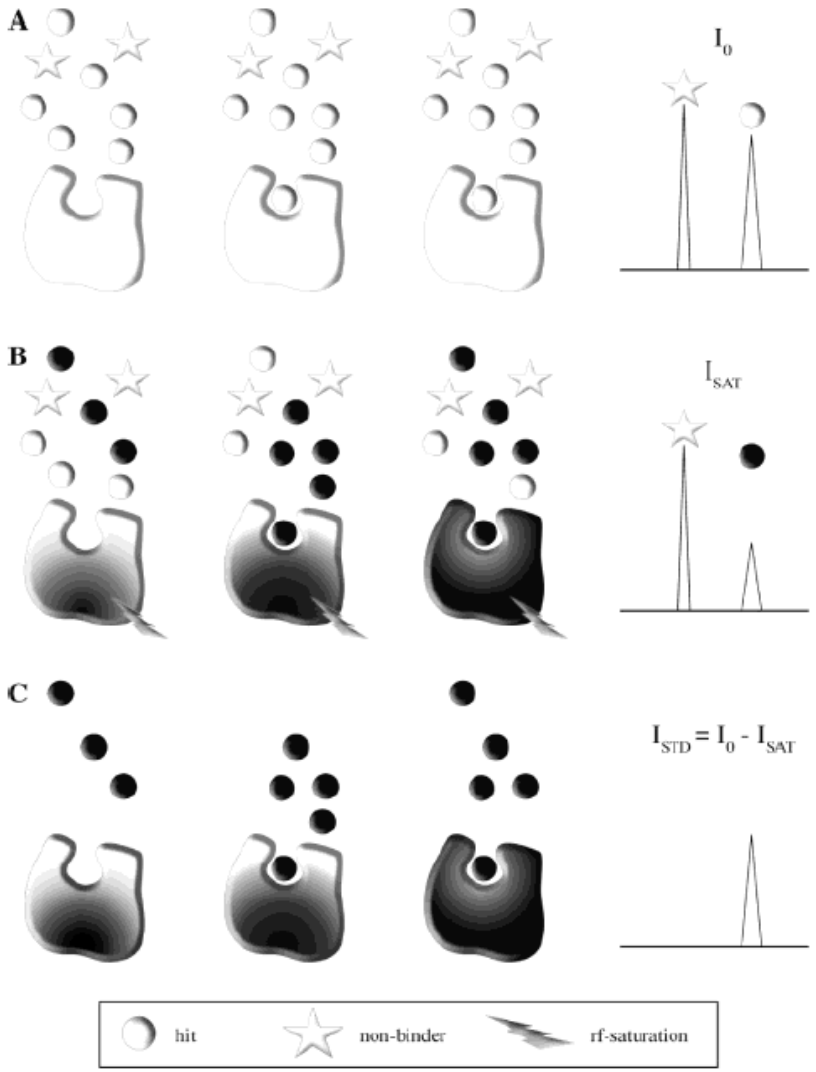
Detection of ligand binding by saturation transfer



- Frequency selective irradiation (lightning bolt) causes selective ^{1}H saturation (shading) of the target receptor. Irradiation is applied for a sustained interval during which saturation spreads throughout the entire receptor via ^{1}H-^{1}H cross-relaxation (spin diffusion).
- Saturation is transferred to binding compounds (circles) during their residence time in the receptor binding site. The number of ligands having experienced saturation transfer increases as more ligand exchanges on and off the receptor during the sustained saturation period.
- Nonbinding compounds (stars) are unaffected.

Lepre et al., Chem.Rev., 2004

STD: saturation transfer difference

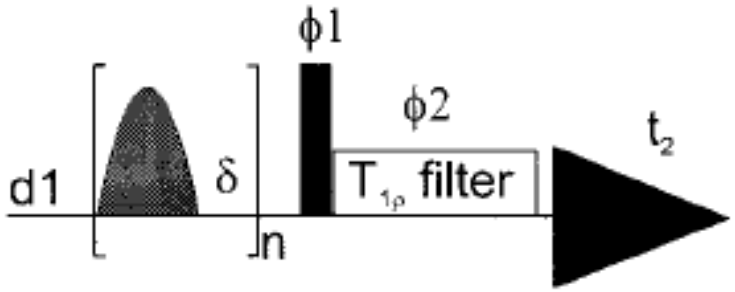


Reference experiment
rf irradiation is applied off-resonance from both the protein and the compounds

On-resonance experiment
rf irradiation selectively saturates receptor and any binding compounds (dark shading)

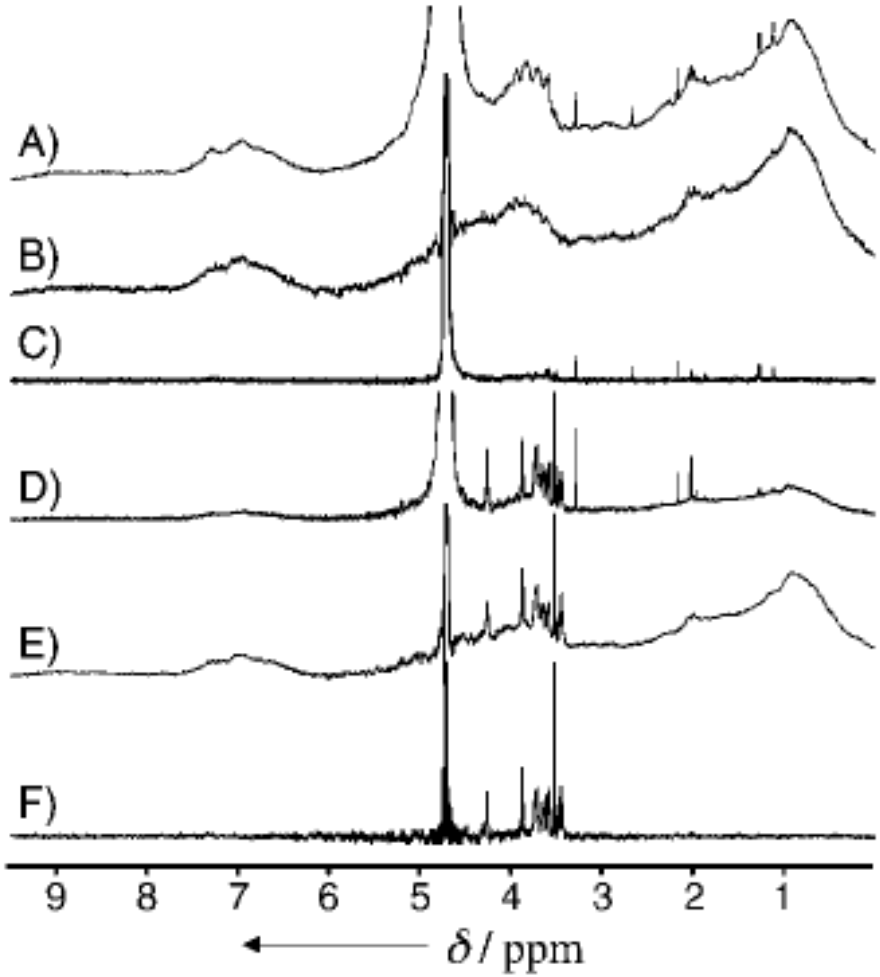
The STD response is the spectral difference $I_{STD}=I_0-I_{sat}$ which yields only resonances from the receptor and binding compounds

STD general pulse scheme



The on and off resonance frequency of the selective pulse is switched between -0.4 ppm and 30 ppm after every scan. The subtraction is performed after every scan by phase cycling.

STD and reference experiments



A) 1D spectrum of a 120 kDa protein (50 µM).

B) STD of P.

C) 1D spectrum of P with $T_{1\rho}$ filter.

D) 1D spectrum of P(40µM) in presence of 1.2 mM ligand without $T_{1\rho}$ filter.

E) STD of P+L, which shows the L signals.

F) STD as in (E) but with the $T_{1\rho}$ filter eliminating all protein background signals.

Meyer and Peters, Angew.Chem., 2003

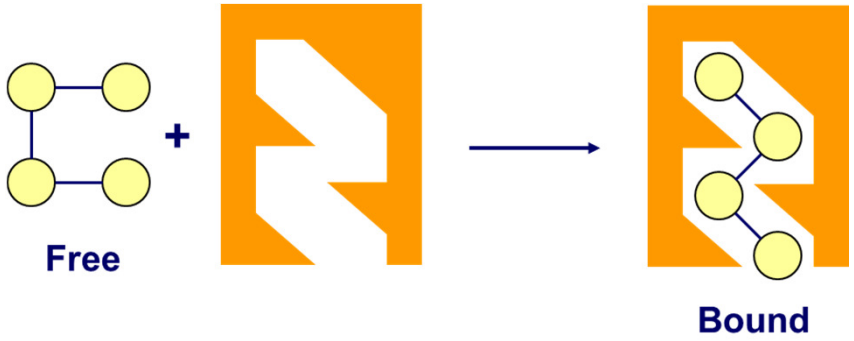
Ligand conformation and TRNOE

• One of the most important things when designing a new drug is to find out how it will bind to its receptor, usually a protein.

• If we have this information we can design new drugs that not only have the chemical requirements for activity that we may know from SAR studies, but which also meet conformational requirements of the binding site.

• One way is to find the structure of the isolated molecule by either X-ray or NMR, and then assume that this is the same conformation we'll see when bound.

• In flexible ligands (99.9% of the interesting stuff…), the change environment (polarity, presence of apolar groups, etc) when going from water to the binding site will most likely change its conformation.



• So, in order to find out the conformation of the ligand when bound, we somehow have to study it either bound to the receptor, or with the receptor present there in some form…

Ligand conformation (continued)

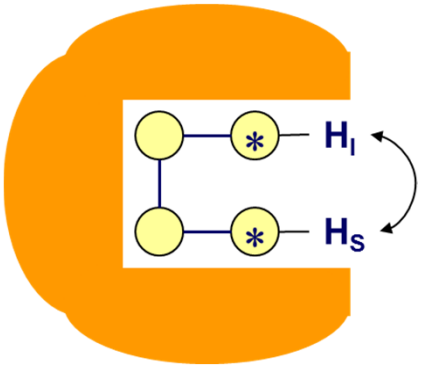
• Depending on the size of the receptor, we can in principle resolve the 3D structure of it plus the ligand.

• There are two problems. First, this is time consuming. After all, we just need the ligand, but if we do it this way we will have to assign the whole protein and compute the structure.

• Second, most receptors are huge, not 10 or 20 KDa, but 100 to 200 KDa, meaning we cannot see anything by NMR. Not only we will have a lot of overlap (even in 3D spectra), but the correlation times are so large that broadening will kill us.

• What in some cases bail us out in this situations are the relative rates of the rise of NOE (cross relaxation) and the binding of the ligand to the receptor.

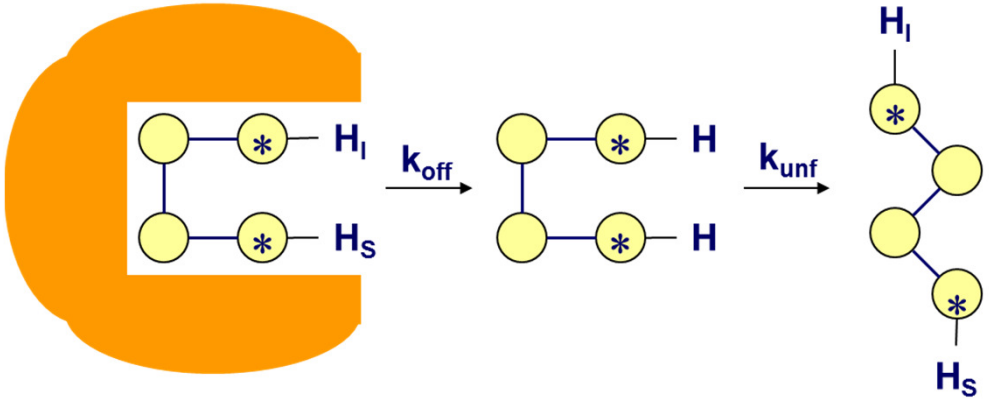
• Say that we have the following ligand/receptor complex:



• When bound, the protons in the marked carbons will have an NOE interaction. It will be very hard to see it with the protein also having tons of other NOE correlations.

Ligand conformation (…)

• Now, say that the ligand dissociates from the complex and goes back to solution. It will adopt its solution conformation in a jiffy:



• Usually, k_{off} (or dissociation constant) is slower than k_{unf} (the 'rate' of unfolding), so we only worry about k_{off}. We define all the constants as follows:

$$K = \frac{k_{on}}{k_{off}} = \frac{[\text{protein-ligand}]}{[\text{protein}]\,[\text{ligand}]}$$

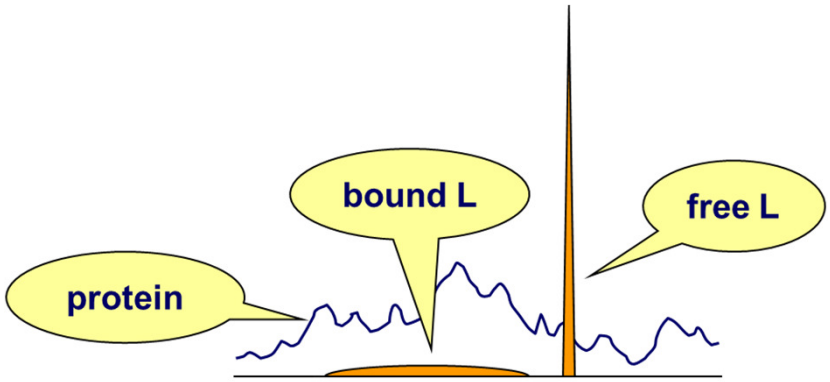
• Irrespective of the k_{off} rate, the NOE interaction between the two protons that appeared in when the ligand was bound will stay for a time that depends on the spin-lattice relaxation rate.

Ligand conformation (…)

• Additionally, if we have good turnover compared to the spin-lattice relaxation rate, we will have several ligand molecules binding to the same receptor before the NOE enhancement of the first one decayed:

• This means that we can do the experiment with an excess of ligand (10 fold or more), and the signals of the ligand will be in larger ratio than 1:1 with those of the receptor (which will be broad and overlapped).

• Another good thing of measuring the NOEs of bound ligands by ***TRNOE*** is that since we will be looking at them in the free molecule, the peaks will be sharp and well resolved:



• Sharp signals are taller (they have the same area), they stand out from all the protein background, and they give far better resolved cross-peaks in a 2D experiment.

Ligand conformation (…)

• If it looks too good to be true, it is too good to be true. We need to meet several criteria to use ***TRNOE***:

  • The ligand cannot bind tightly to the receptor (we need constant exchange between bound and free ligand).

  • The $\mathbf{K_{off}}$ rate has to be much smaller than the spin-lattice relaxation rate, otherwise the NOE dies before we can detect it.

Summary

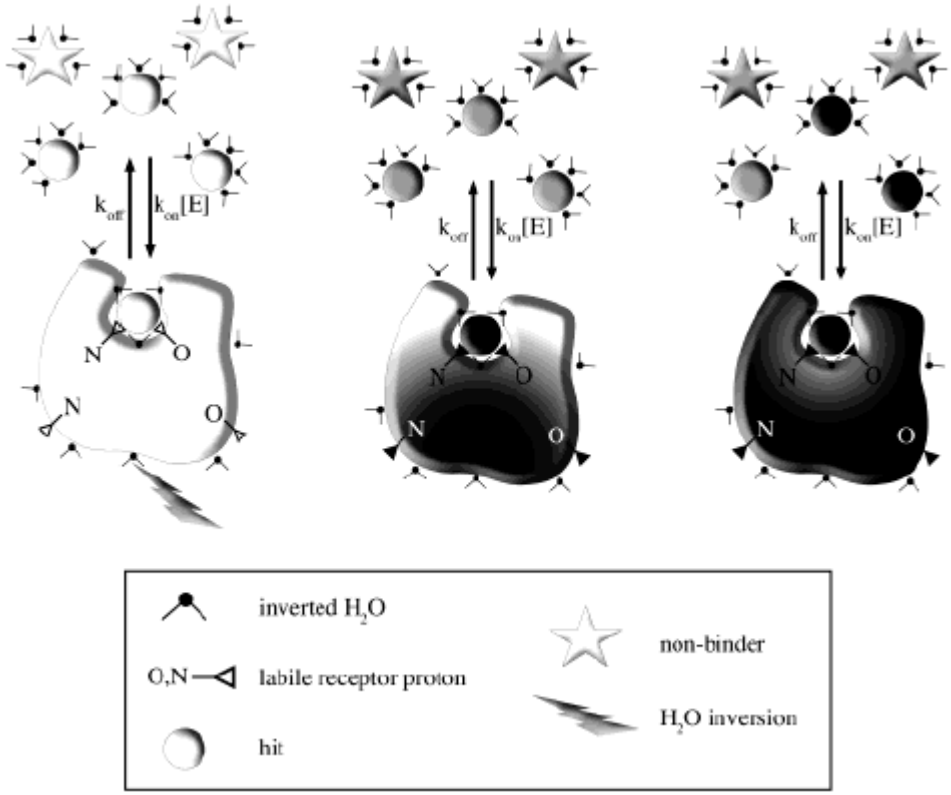
• With NMR we can study dynamic processes that happen at rates slower than the NMR timescale. We can obtain rate constants and $\mathbf{\Delta G^{\ddagger}}$ values for dynamic processes.

• TRNOE is a variation of the NOE experiment in which we can look at the NOE enhancements of molecules bound to a large receptor.

• Although there are several conditions that need to be met, we don't have to worry about the size of the receptor.

Ligand-based experiments
Cross-relaxation

Water-LOGSY (water-ligand observed via gradient spec.)

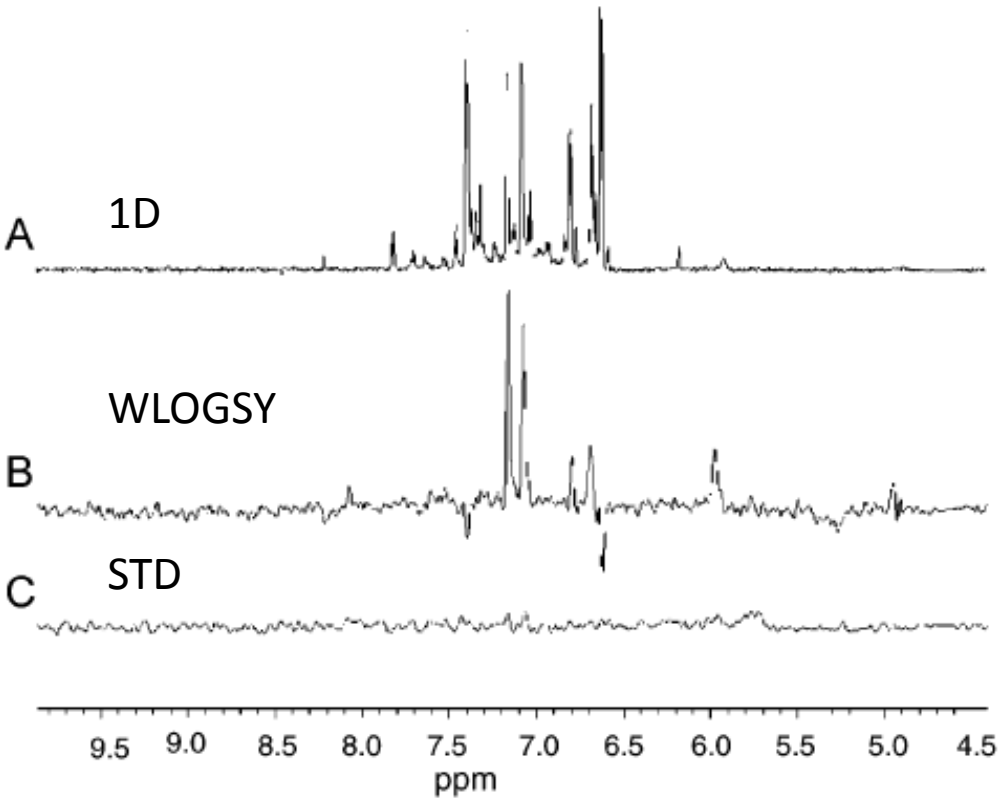


Water
↓
Receptor
↓
Ligand

Magnetization transfer from bulk water to ligand occurs via labile receptor protons within and remote from the ligand-binding site as well as from long-lived water molecules within the binding pocket. Dark gray and light gray shading indicate magnetization transfer from inverted water to ligand protons in the slow tumbling (i.e. receptor-ligand complex) and fast tumbling (i.e. free ligand) limits, respectively. Only the hits experience both types of magnetization transfer.

Lepre et al., Chem.Rev., 2004

Water-LOGSY: hit identification



Binders: negative cross-relaxation rates due to long τ_c

Non-binders: positive cross-relaxation rates due to short τ_c

Result: peaks of opposite sign

Performs better than STD for nucleic acids, having poor spatial proton density

Lower limit is K_d~0.1μM

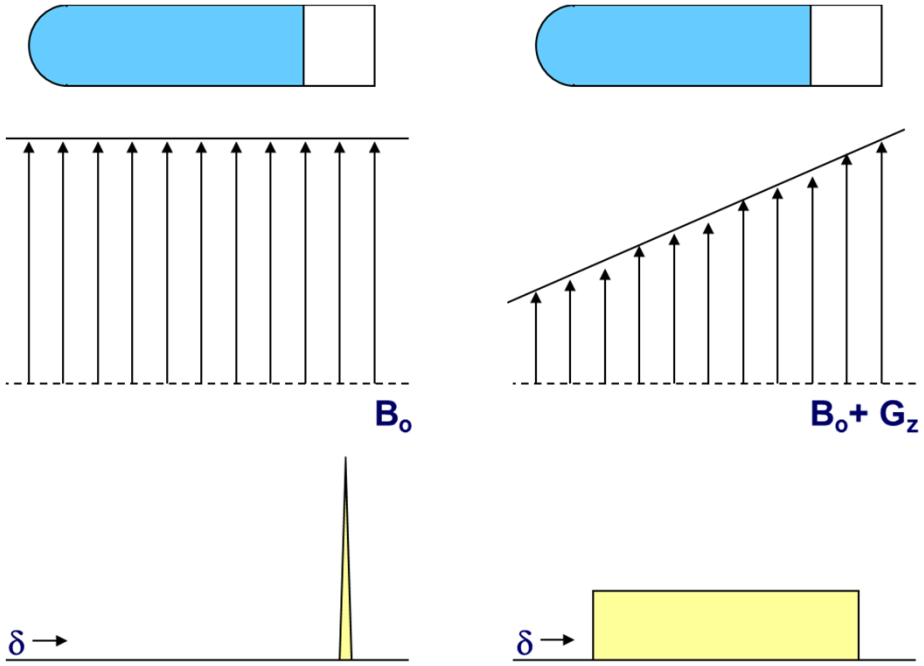
Lepre et al., Chem.Rev., 2004

Ligand-based experiments
Translational diffusion

Magnetic field gradients and diffusion

• So far all our discussions have dealt with 'perfect' magnetic fields (i.e., homogeneous $\mathbf{B_o}$). We obviously want this for good resolution and sensitivity. However, creating a gradient of known characteristics on $\mathbf{B_o}$ can be extremely useful.

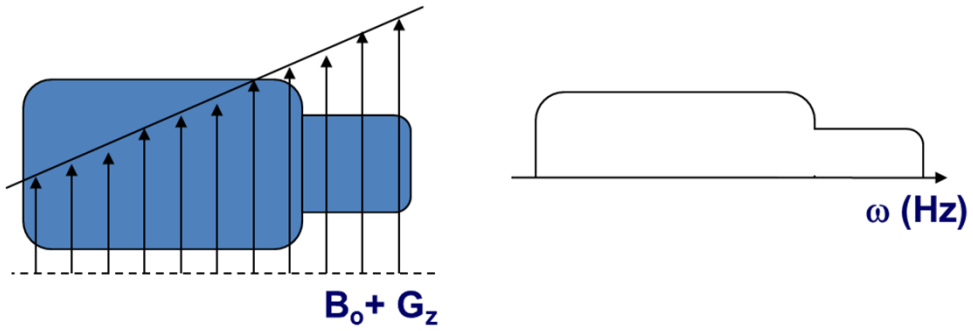
• A gradient in the magnetic field results in different **B**s. If we just consider a linear variation along the **z** axis (i.e., a **z-gradient**, $\mathbf{G_z}$) and a sample of water, what we'll see is that water molecules at different positions along **z** will have different δs (because $\delta \propto \gamma (\mathbf{B_o} + \mathbf{G_z})$):



• Notice that the signal is proportional to 'sample mass'…

Magnetic field gradients (continued)

• So, how is this useful? For starters, we can get an image of the sample if we apply the gradient during acquisition. Since spins at different positions along the tube have different δs, we get a 'continuous' spectrum that parallels the shape of the container (MRI).



• In other words, with a linear gradient the spins end up ***spatially encoded***. This means that we 'know' to what part of the tube (or "arm") the spin belongs based on the gradients that we applied. Add contrast based on different relaxation times for different tissue, and you get MRI.

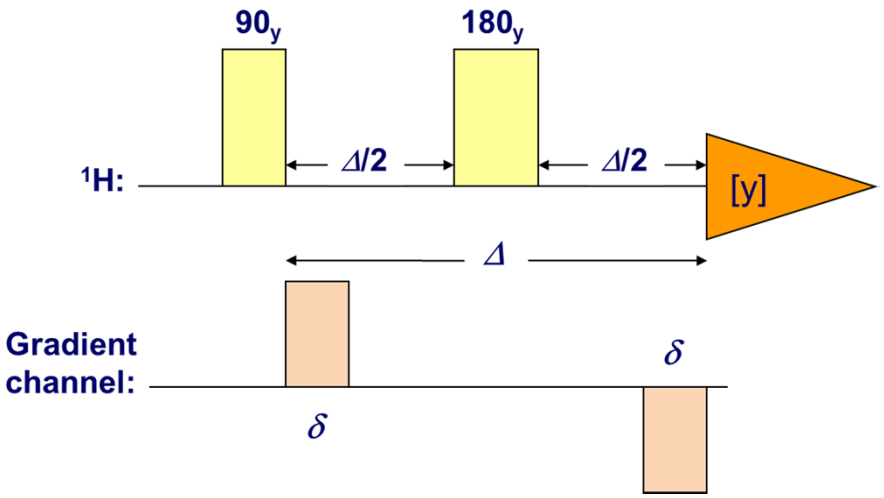
• In addition, by combining gradients of different signs we can spatially encode the nuclei, allow them to 'evolve' (times, pulses, etc.), and then decode them.

• Spins that did (or did not) behave as expected during the evolution period will (will not) show in the spectrum…

Gradients and diffusion

• Which brings us to diffusion measurements. Measuring ***self-diffusion coefficients*** (***D***s) is extremely important in chemistry and biology. It tells us about interactions between molecules, their motions, etc., etc.

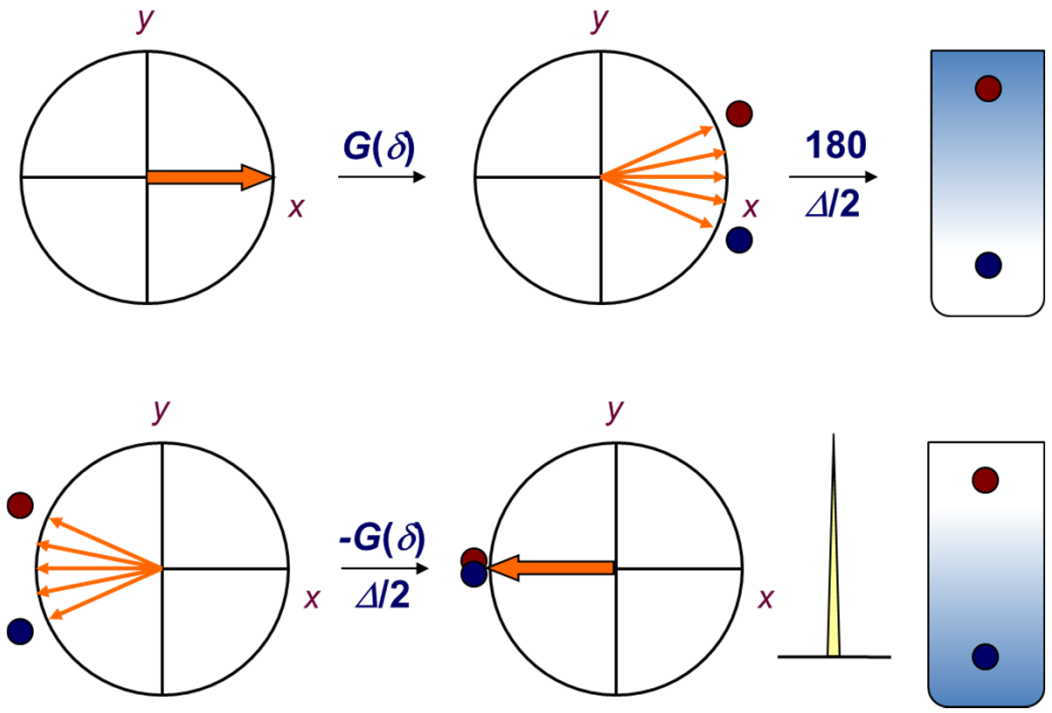
• Pulse-field gradient NMR is ideal to measure the diffusion of particles bearing NMR-active nuclei. The most basic technique involves combining a spin-echo with two gradients of opposite sign and length δ separated by a delay Δ:



• We have to analyze how the spin-echo will look like under the effect of the encoding and decoding gradients for nuclei that diffuse at different rates. Keep in mind that the gradient will make things move faster/slower in the rotating frame (i.e., change their 'chemical shift).

Gradients and diffusion (continued)

- For a spin that does not diffuse much (after the 90):

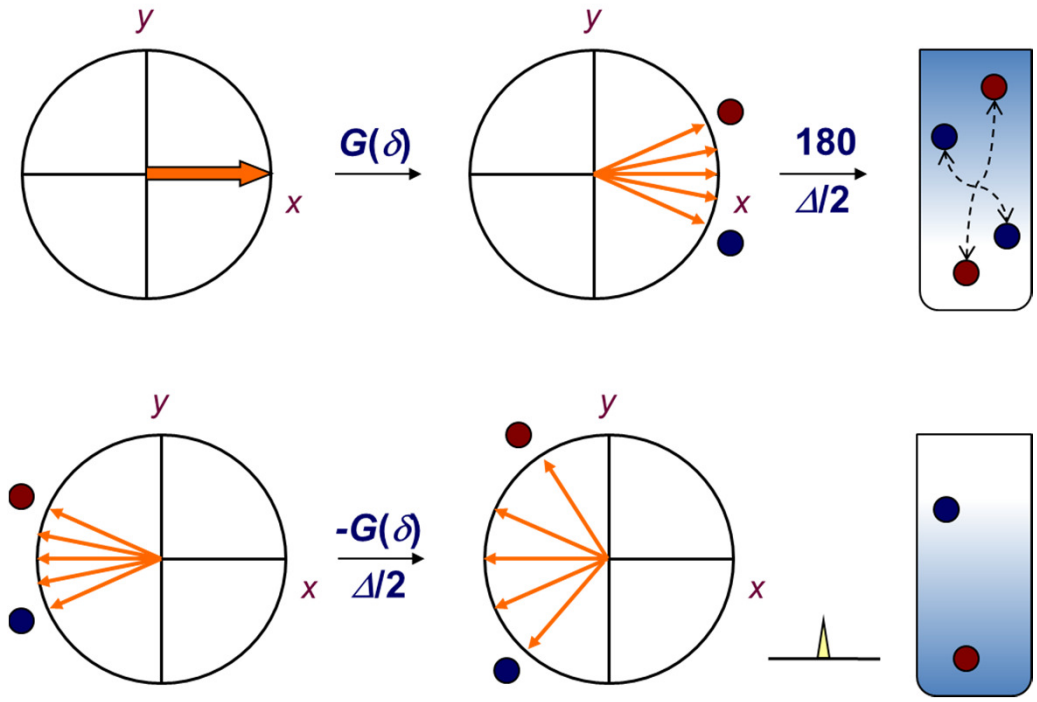


- Here the blue/red dots represent the same type of nuclei in different regions of the ‘tube’…

- Basically, since the spin is not moving the gradients have little effect. That is, the spin did not move from the area it was, so the decoding gradient had the same and opposite effect as the encoding gradient. In other words, the signal intensity for this nuclei did not change…

- Things will change quite a bit if we have a fast-moving spin…

Gradients and diffusion (continued)



• In this case, since the nuclei moves away from where it was, the decoding gradient has a completely different effect (i.e., things will dephase further). Therefore, the signal intensity will be greatly attenuated.

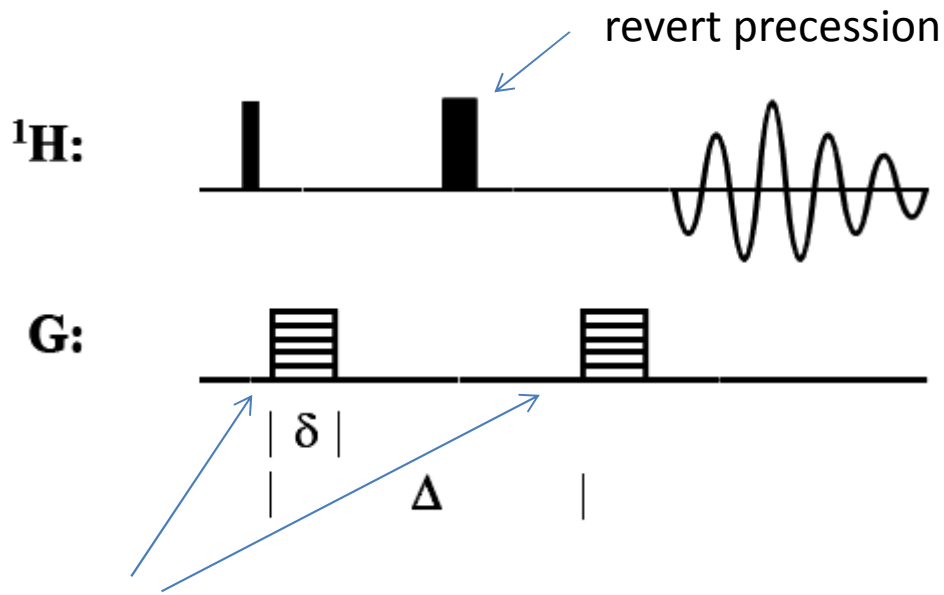
• The end results is that the the faster the nuclei diffuse, the smaller their signals will be in the spectrum. By selecting the values of ***G***, δ, and Δ we can 'fine-tune' the experiment for species with self-diffusivities going down to 10^{-12} m^2s^{-1} (this is for our Bruker).

Measuring translational diffusion

$$D = \frac{kT}{6\pi\eta r_s}$$

Stokes-Einstein, diffusion depends on size and shape of molecules

k: Boltzmann constant; η: viscosity of the liquid; r_s: hydrodynamic radius



spatial encoding/decoding

δ: 0-10ms, Δ: 1-1000ms, g: up to 20 T m^{-1}

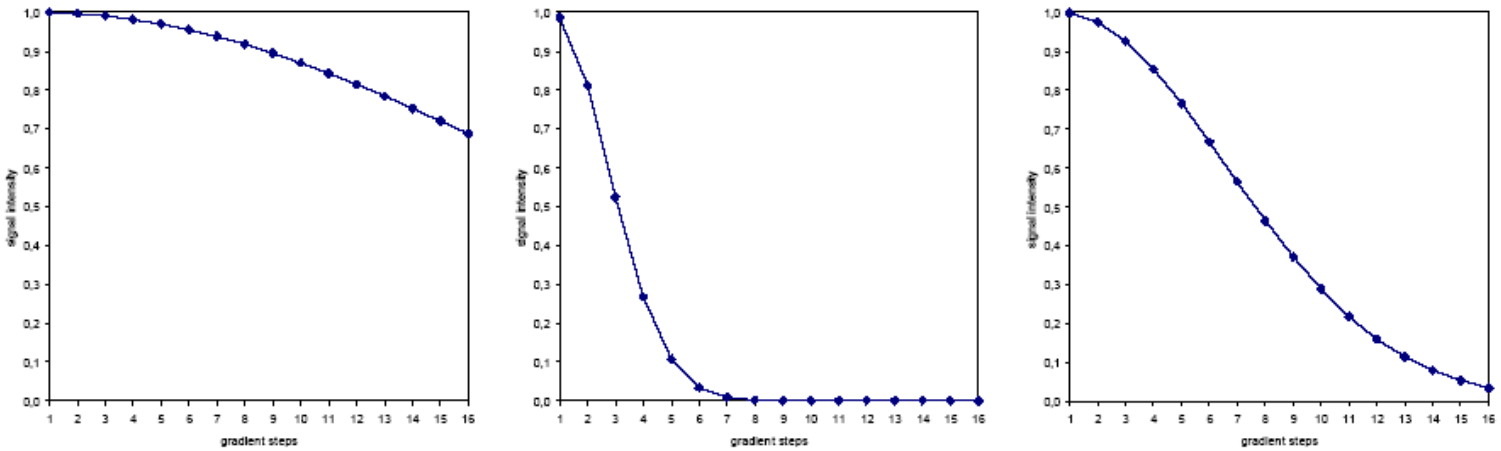
PFG-SE
pulsed field gradient spin echo

Motion is measured by evaluating the attenuation of a spin-echo signal.

Attenuation is achieved by the dephasing of nuclear spins due to a combination of the translational motion and the imposition of gradient pulses.

Brand et al., Prog.NMR Sp., 2005

Measuring translational diffusion



Simulated diffusion decay curves by varying the gradient strength from 2 to 95 % in 16 steps for the same diffusion constant, but with different selection for Δ *and* δ*. They are chosen too small (A), too big (B), and properly (C) to sample data points along the whole decay curve.*

$$I = I_0 e^{-D\gamma^2 g^2 \delta^2 \left(\Delta - \frac{\delta}{3}\right)}$$

non-linear least squares fitting to extract D

Multiple site binding

Two non-identical non-independent binding sites, 1:2 stoichiometry

L is added to P, P is observed; slow exchange is assumed

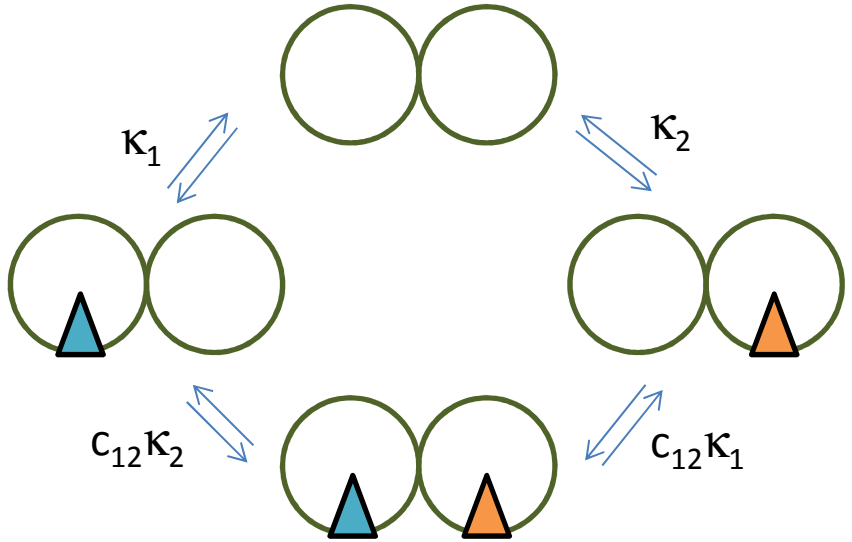
Rigorous method: no assumption needs to be made, requires solving cubic equation

$$P=C_P/[1+(\kappa_1+\kappa_2)L+c_{12}(\kappa_1+\kappa_2)L^2]$$

$$PL'=P\times\kappa_1\times L$$

$$PL''=P\times\kappa_2\times L$$

$$PL_2=P\times c_{12}\kappa_1\kappa_2\times L^2$$



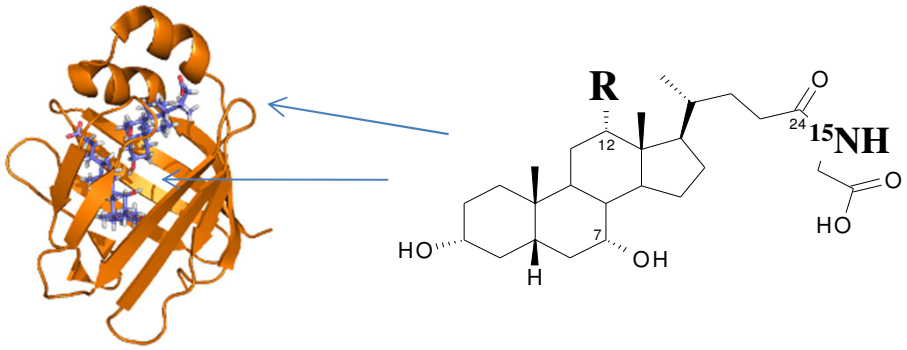
κ_1 and κ_2: intrinsic affinity constants

c_{12}: intrinsic cooperativity factor

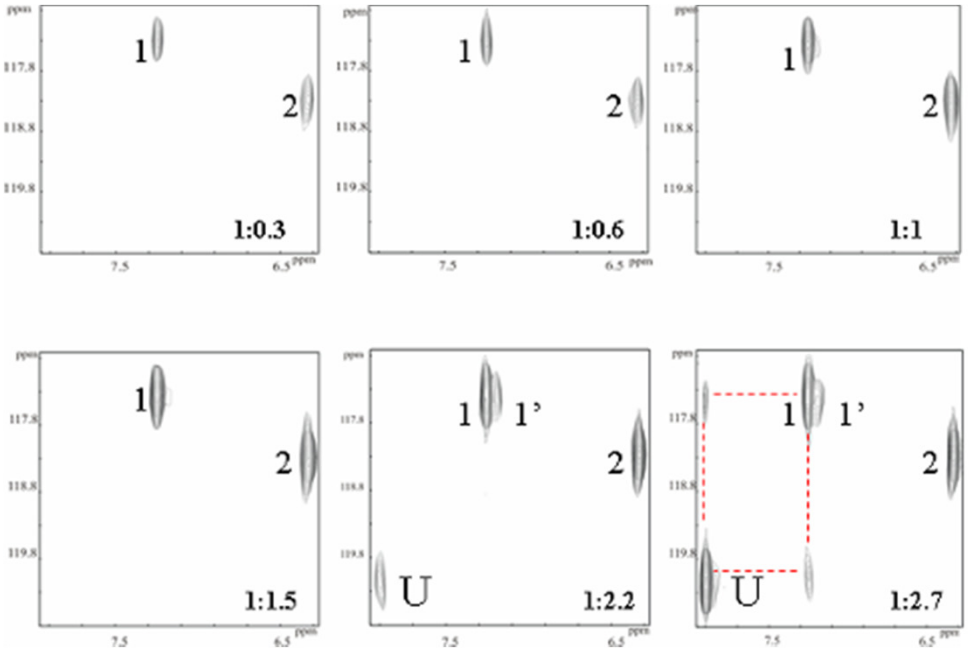
$$c_{12}\kappa_1\kappa_2\times L^3+[(2C_P-C_L)c_{12}\kappa_1\kappa_2+\kappa_1+\kappa_2]\times L^2+[(C_P-C_L)(\kappa_1+\kappa_2)+1]\times L-C_L=0$$

Di Cera, Thermodyn. Theory of Site-Specific Binding, Cambridge U.P., 1995; Tochtrop et al., PNAS, 2002

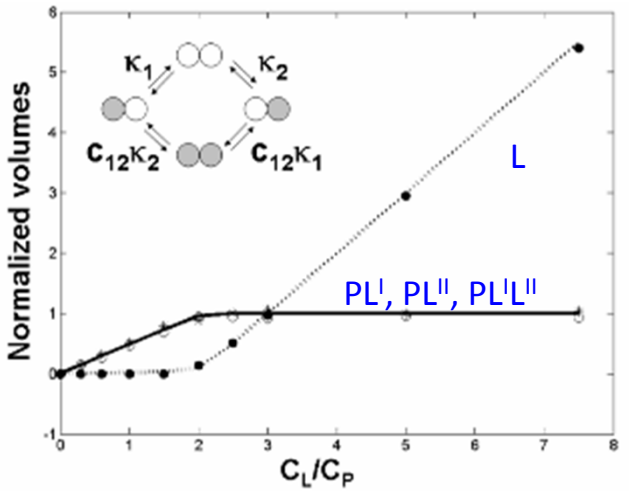
Two non-identical non-independent binding sites, 1:2 stoichiometry



R=H → GCDA
R=OH → GCA



NMR titration – ligand observed



C_{12}>1000 !

Pedò et al., Proteins, 2009