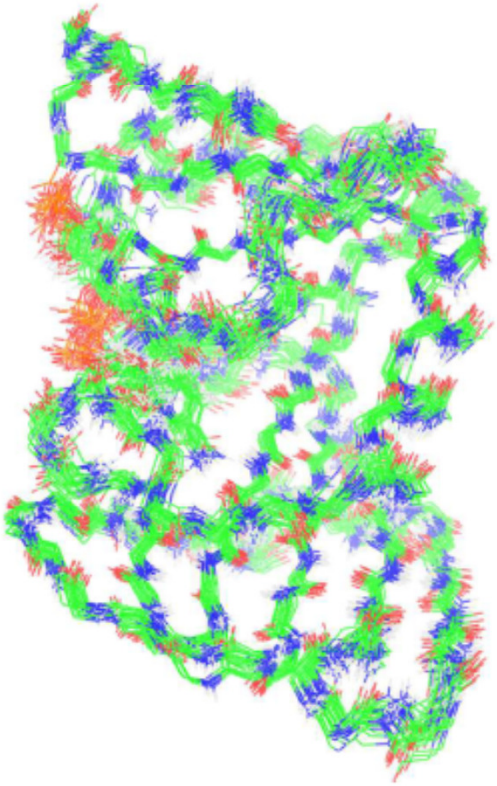
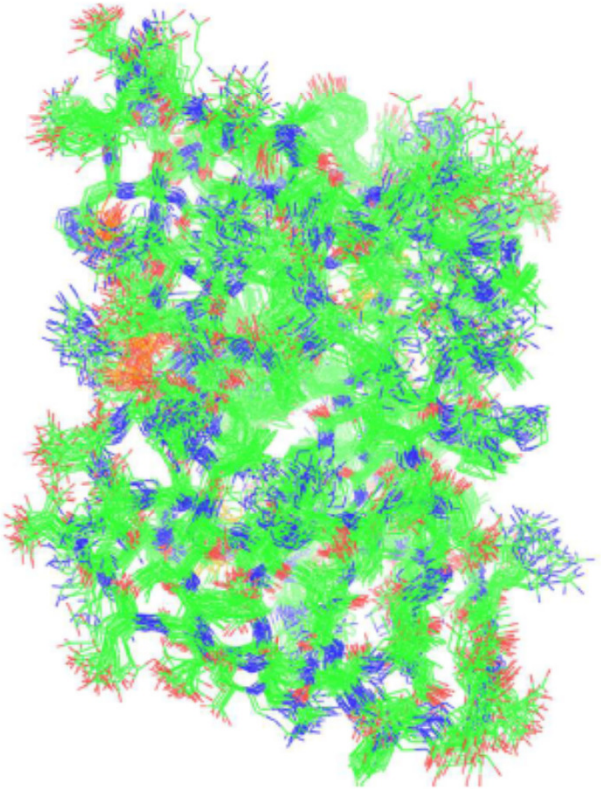
Structure solved in solution by NMR:

Proteins exist as conformational ensembles

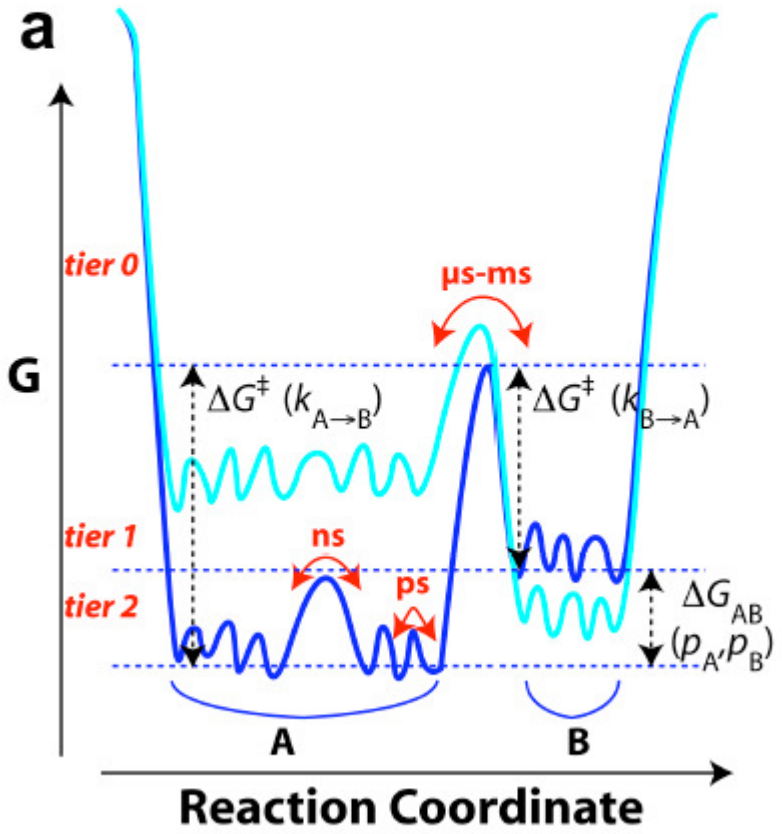


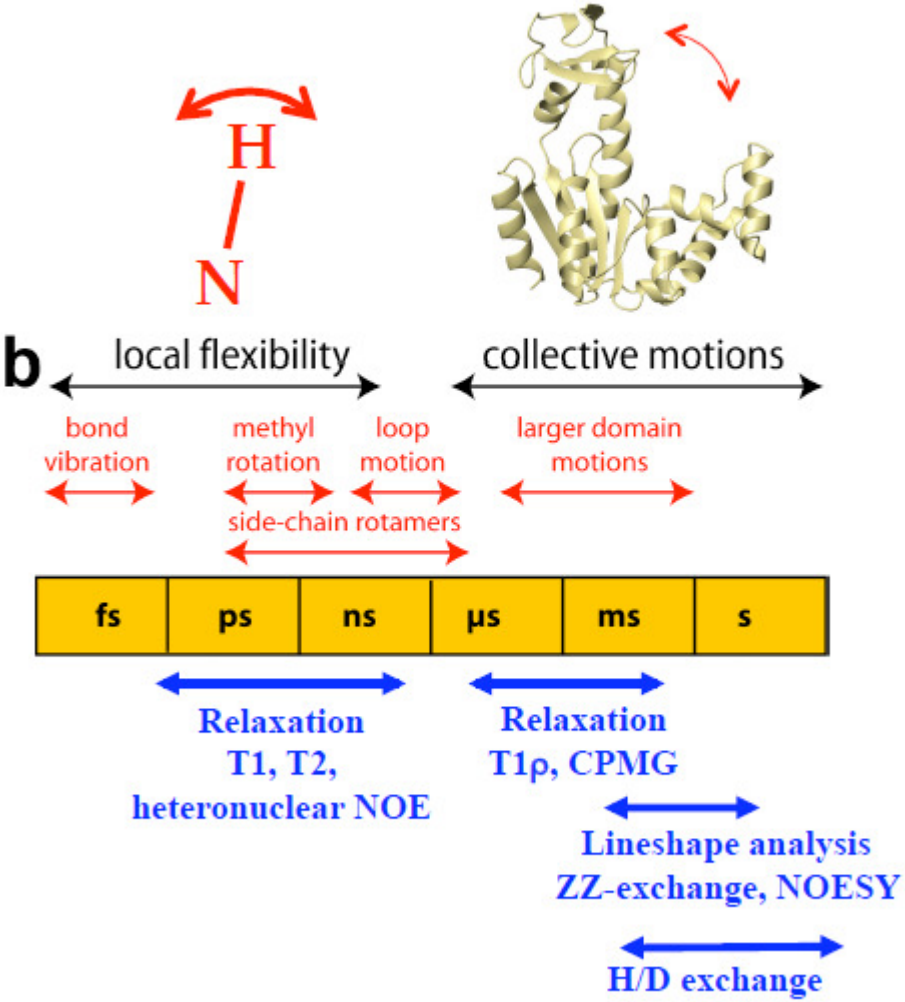
backbone atoms only



all atoms (i.e. with side chains)

Protein Dynamics by NMR





Key Points

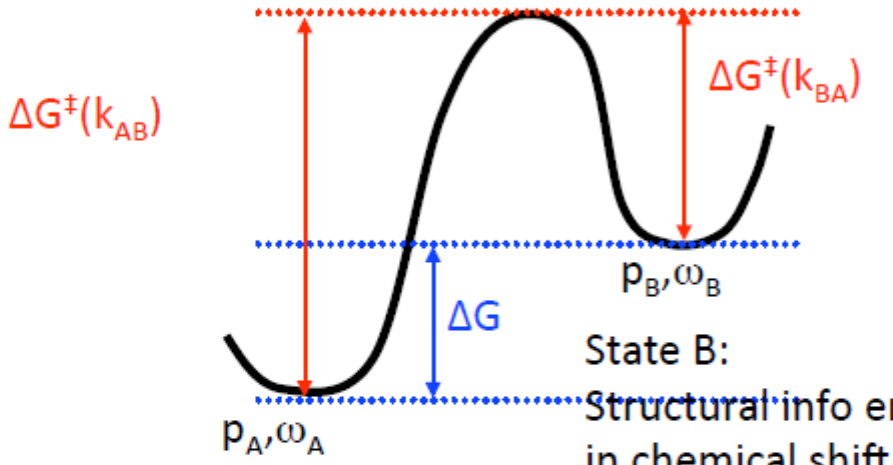
- NMR dynamics divided into 2 regimes: fast and slow.
- How protein motions affect NMR parameters depend on whether they are faster or slower than the rotational correlation time
- Fast timescale dynamics (ps-ns)
  - limited by rotational correlation time of protein
  - parameters describe distribution of states
- Slower timescale dynamics (µs-ms)
  - require chemical shift difference
  - measured more directly

Slow Timescale Exchange

- Great for characterizing a 2-state process:
  - Open vs closed conformation of a protein
  - Free vs bound state
  - Folded vs unfolded
- 3 or more state
  - More challenging to analyze quantitatively
- Strength of NMR
  - Simultaneously measure
    - exchange rate (kinetics)
    - populations (thermodynamics)
    - chemical shifts (structural information)
  - Site-specific resolution.
    - Is the process global or local?
    - If global, all residues should have the same exchange rate and populations

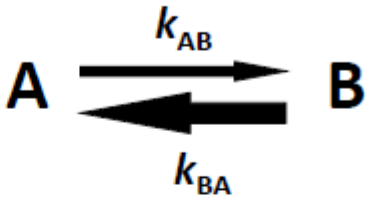
****Fast and slow regimes within the slow-timescale limit!!!****

Characterizing a 2-state processes



State A:
Structural info embedded
in chemical shift, ω_A

State B:
Structural info embedded
in chemical shift, ω_B



$$k_{ex} = k_{BA} + k_{AB}$$

Thermo:

$\Delta G = -RT \ln K_{eq}$

$K_{eq} = p_B / p_A$

Kinetics:

$k_{ex} = k_{BA} + k_{AB}$

$k_{AB} = p_A k_{ex}$

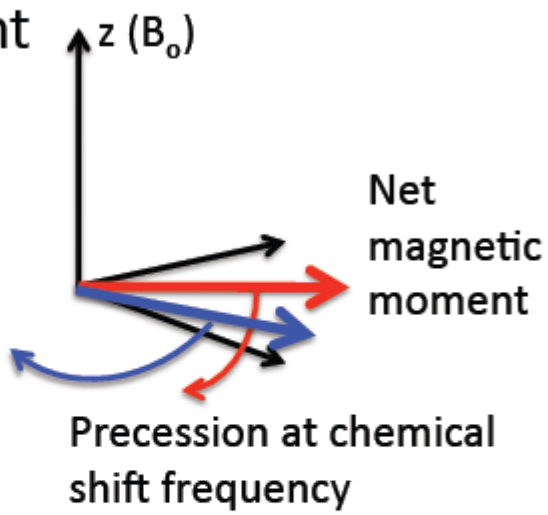
$p_A + p_B = 1$

$p_A > p_B$

$k_{BA} > k_{AB}$

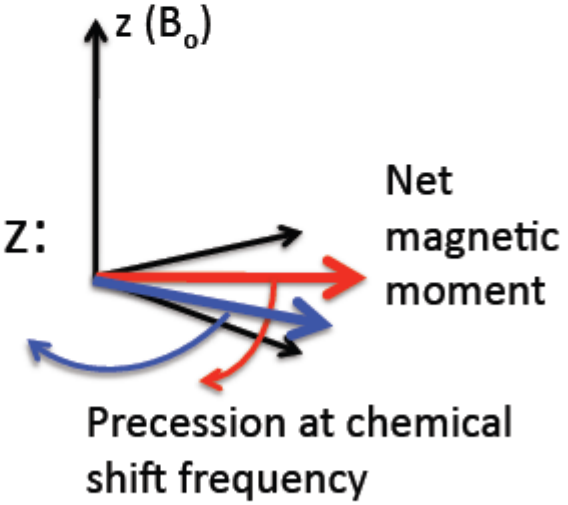
Basic NMR reminders

- Chemical shift depends on the environment of the nucleus
  - Hydrogen bonding, secondary structure, ring currents, electrostatics, side chain torsion angles, etc.
  - Two different states have two different chemical shifts
- Integrate to determine populations
  - Just like organic chemistry – integrate peaks to see which correspond to 1,2, or 3 protons.
  - If two peaks correspond to the same nucleus in two states, then the integrals give the relative populations
  - If peaks are of similar width, can use peak height instead of volume.

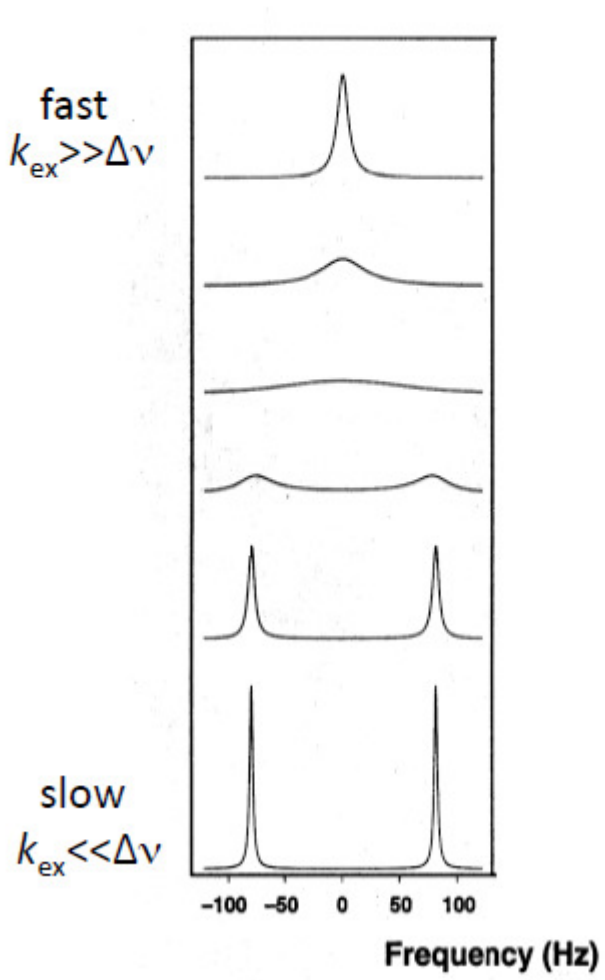


Basic NMR reminders

- Chemical shift is a precession ***frequency***.
  - Serves as a reference point for measuring protein dynamics.
  - Slow timescale dynamics are described as fast or slow depending on the relative values of k_{ex} and $\Delta\omega$ (chemical shift difference between the two states).
- To convert from $\Delta\nu$ in ppm to $\Delta\nu$ in Hz:
  - use $\nu=\gamma B_o/2\pi$ $\quad(\nu=\omega/2\pi)$
  - ppm is parts per million, so 1ppm=600Hz on a 600MHz spectrometer.



Theoretical NMR line-shapes for two-site exchange



k_{ex} (s^{-1})
10000
2000
900
200
20
< 0.1

$$A \underset{k_{BA}}{\overset{k_{AB}}{\rightleftharpoons}} B$$

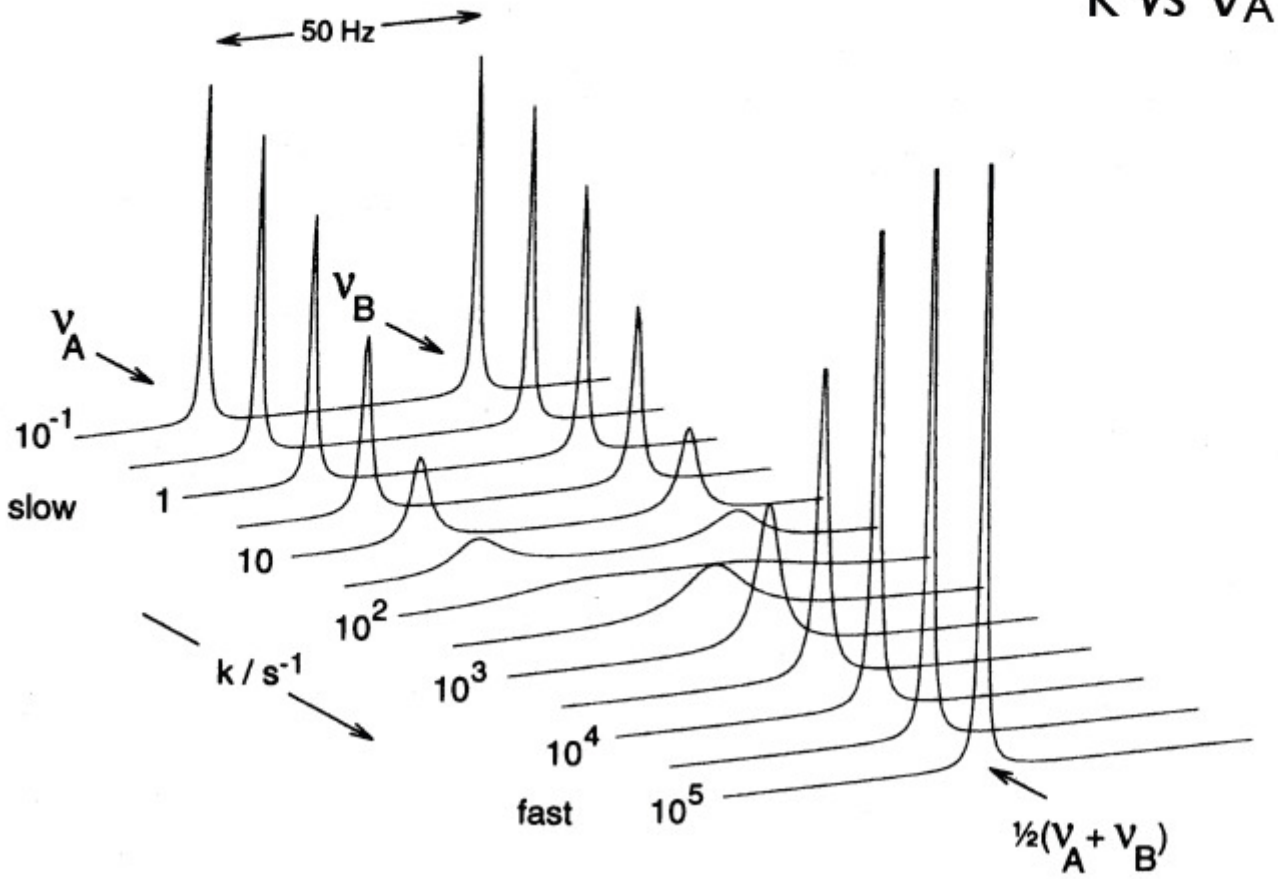
$$k_{ex} = k_{BA} + k_{AB}$$

Remember: Chemical shift difference (Δν) is a frequency.

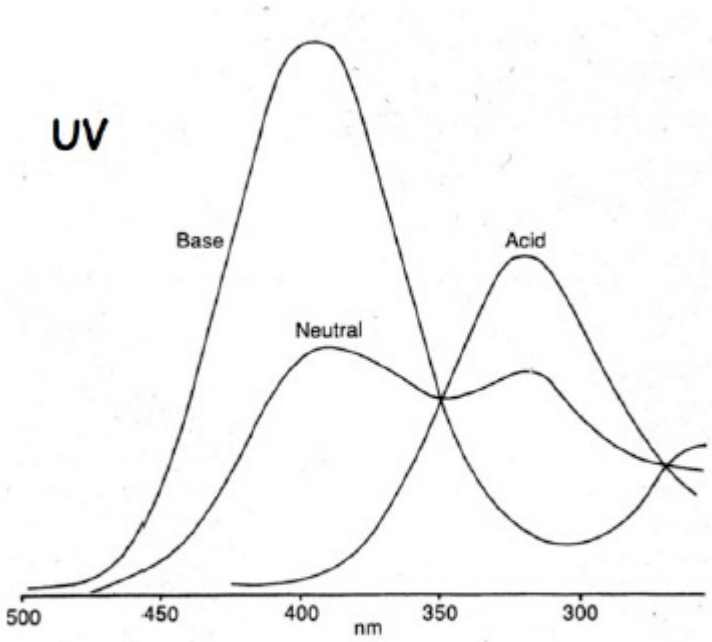
NMR Spectroscopy - Protein dynamics

Chemical exchange

k *vs* $\nu_A - \nu_B$

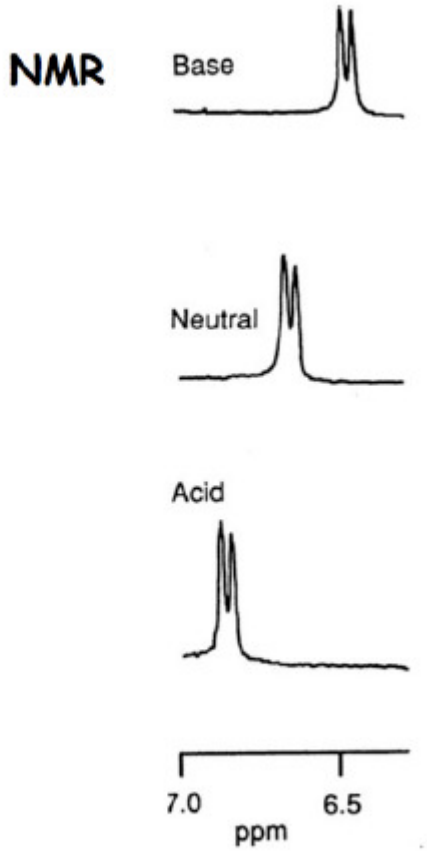


NMR vs UV time scales



$\Delta\nu = 1.8\ 10^{14}$ Hz

$\nu\lambda = c = 3 \times 10^{8}\ ms^{-1}$



$\Delta\nu = 100$ Hz

Lineshape Analysis

- Requires a titration
  - Theoretical equations for lineshape (including peak width, intensity, separation)
  - Parameters: intrinsic relaxation rates (R_2^o), exchange rate (k_{ex}), populations (p_A), chemical shift difference ($\Delta\nu$)
  - Change conditions so that the values of the parameters change in distinct ways so they can be deconvoluted
- Fit the lineshape as a function of titration
  - Adding ligand (free vs bound)
  - Adding denaturant (folded vs unfolded)
  - Changing pH (pKa determination)
  - Changing temperature (folding, conformational change, almost any process)

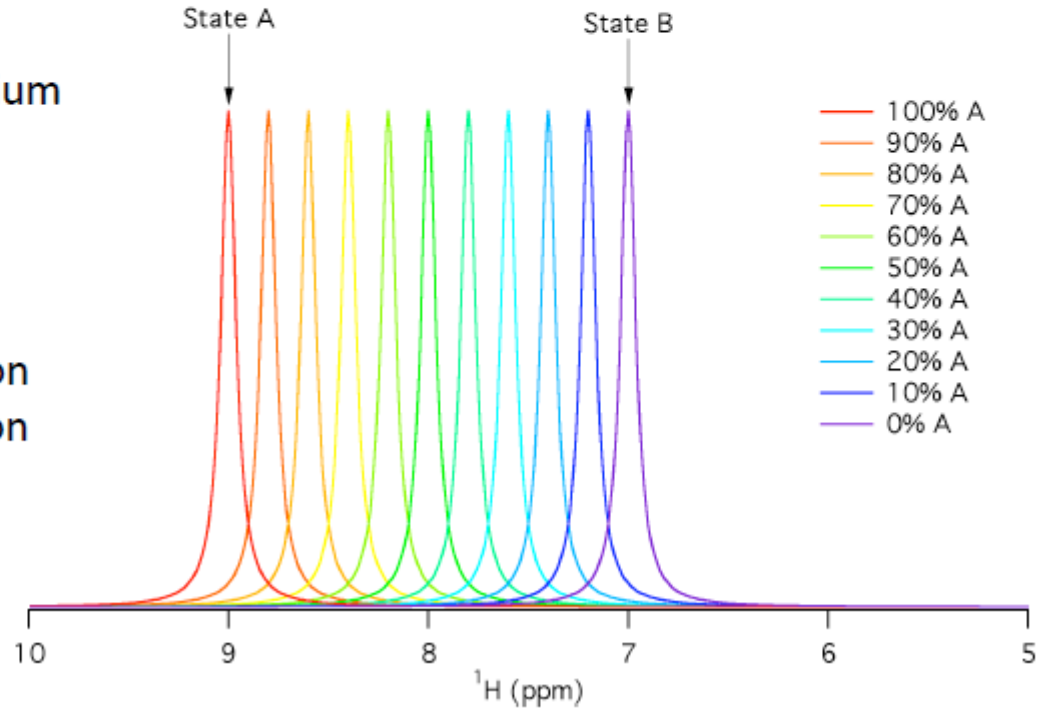
Fast Exchange $k_{ex}>>\Delta\nu$

- Observe a single peak at the weighted-average position.
- What happens upon titration?
- As population shifts, peak positions shifts

Ex. Shifting 2-state equilibrium
•State A=free
State B=bound
•State A=folded
State B=unfolded
•State A=open conformation
State B=closed conformation

If 600 MHz spectrometer,
Then $\Delta\nu$=1200 Hz

k_{ex}?



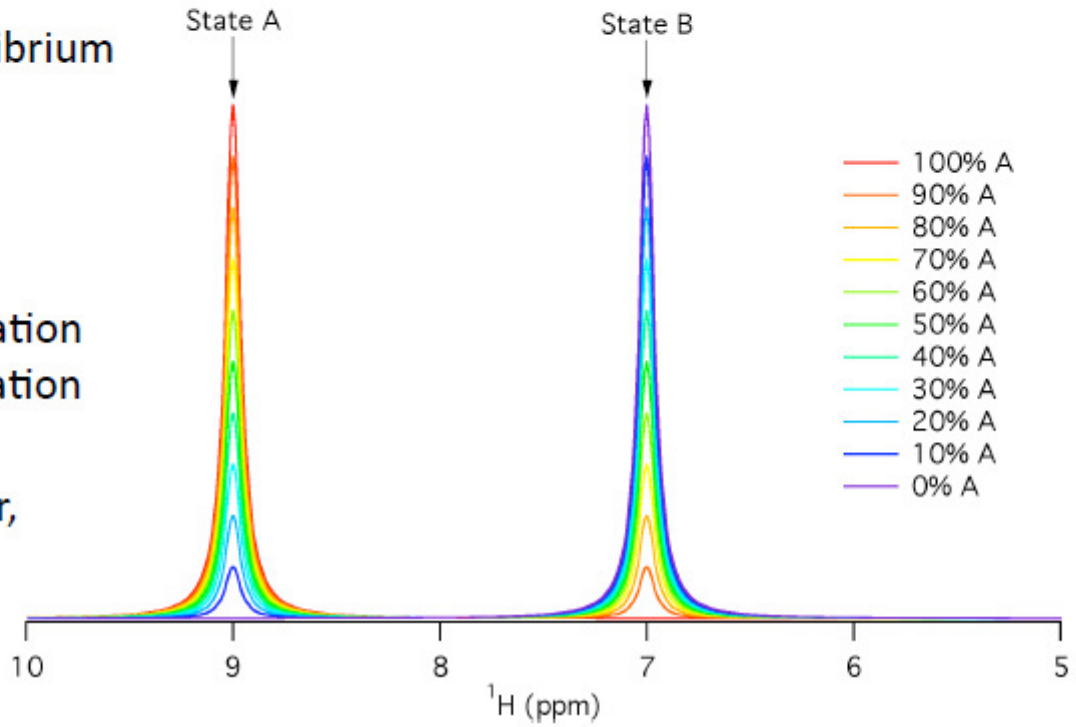
Slow Exchange $k_{ex} << \Delta\nu$

- Peaks don't shift.
- What happens upon titration of a 2-state system?
- As population shifts, peaks corresponding to each state appear/ disappear

Ex. Shifting 2-state equilibrium
•State A=free
State B=bound
•State A=folded
State B=unfolded
•State A=open conformation
State B=closed conformation

If 600 MHz spectrometer,
Then $\Delta\nu$=1200 Hz

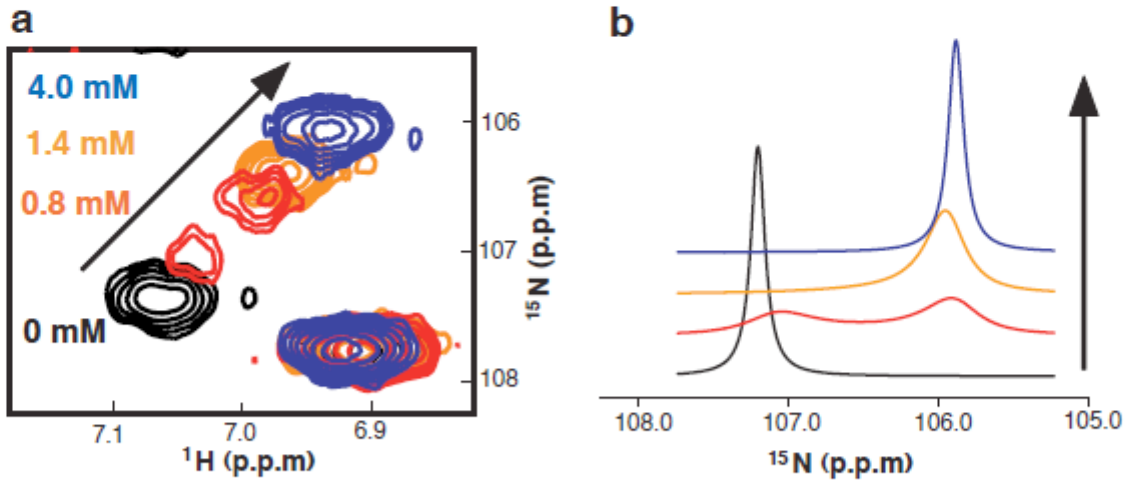
k_{ex}?



Often somewhere in the middle

- k_{ex} and $\Delta\omega$ both influence the peak position/lineshape. Careful analysis can determine p_A, k_{ex}, and $\Delta\omega$.
- $\Delta\omega$ will not be the same for all residues in a protein – might have some in slow exchange, some in fast exchange, some in intermediate.

Wolf-Watz, *et al.* *Nat. Struct. Mol. Biol.* **11**, 945-949.

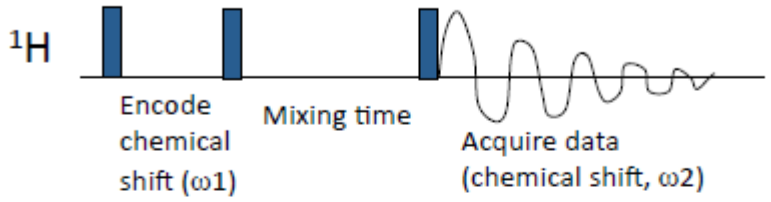


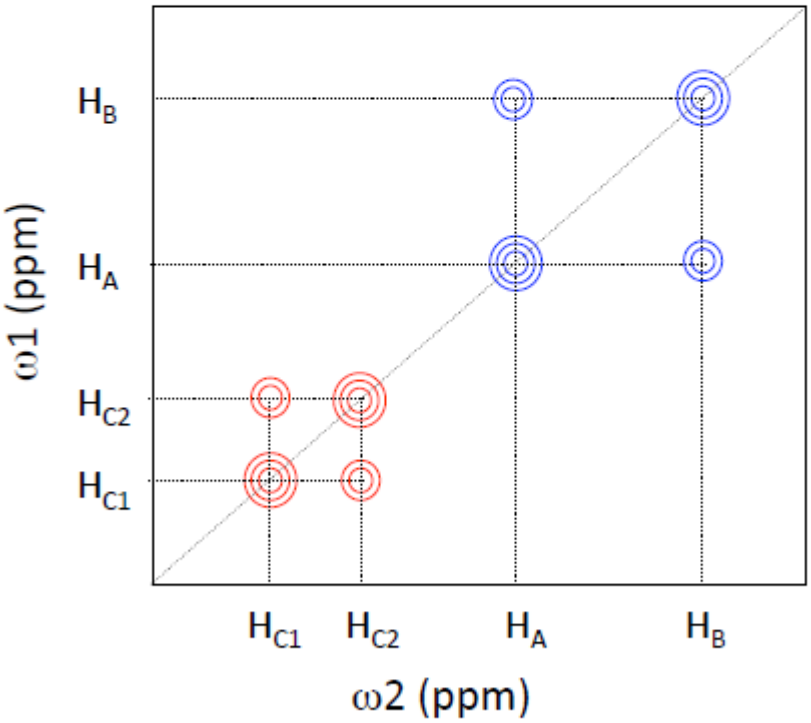
Titration of ligand (concentration indicated) into protein. A) shows a peak from the protein HSQC undergoing the transition from free to bound. B) shows the 1D lineshape simulation of the spectrum.

NOESY/ZZ analysis

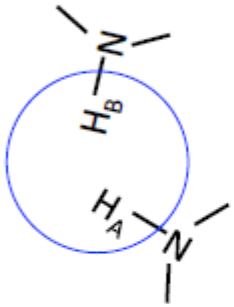
- Basic strategy: Record initial chemical shift, wait (mixing time), record final chemical shift
  - No exchange: initial and final states are the same ($\omega_1=\omega_2$) – observe diagonal/auto peak
  - With exchange: chemical shift of final state is different than the initial state – observe crosspeak ($\omega_1\neq\omega_2$)
  - NOTE: chemical shift of the two states must be different in order to observe the exchange
    - Ex. regions close to binding interface will show the effect of binding, regions far away will not.
    - By chance, even if there is a conformational change, an individual residue within that region may have the same chemical shift in both states.
- Useful for
  - Slow exchange, 2sets of peaks already visible in spectrum
  - Exchange rates 10s-100s of milliseconds
- Direct kinetic measurement – vary mixing time and see how much conformational change occurs

NOESY Analysis

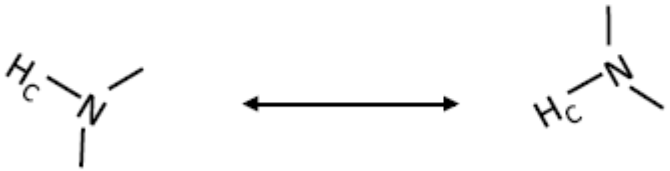




Crosspeaks arise when:



2 protons are close in space



State 1 State 2

Slow conformational exchange between 2 states with different chemical shifts

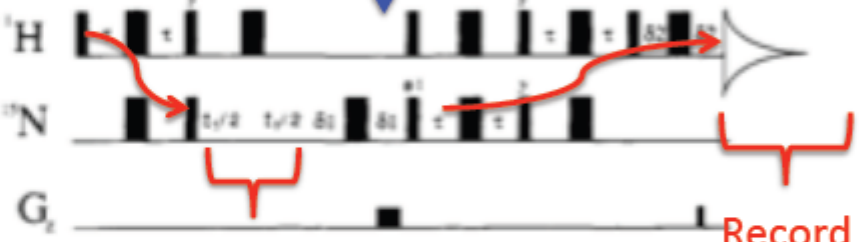
ZZ-exchange

Insert mixing time (spins along z, hence the name ZZ-exchange)

Transfer magnetization from 1H to 15N

Transfer magnetization from 15N to 1H

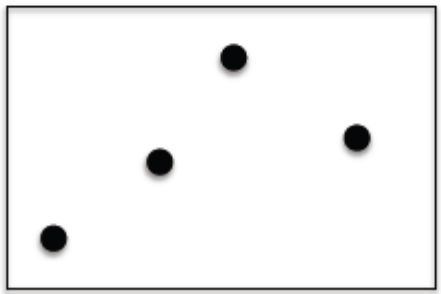
HSQC



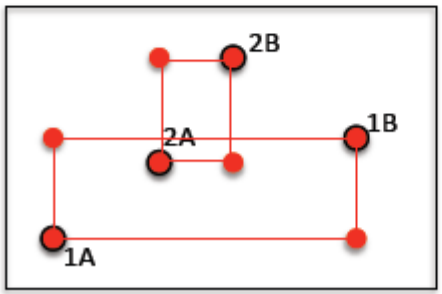
Record chemical shift in indirect dimension (t_1), ^{15}N frequency in this case

Record chemical shift in directly detected dimension (t_2), ^{1}H frequency in this case

HSQC

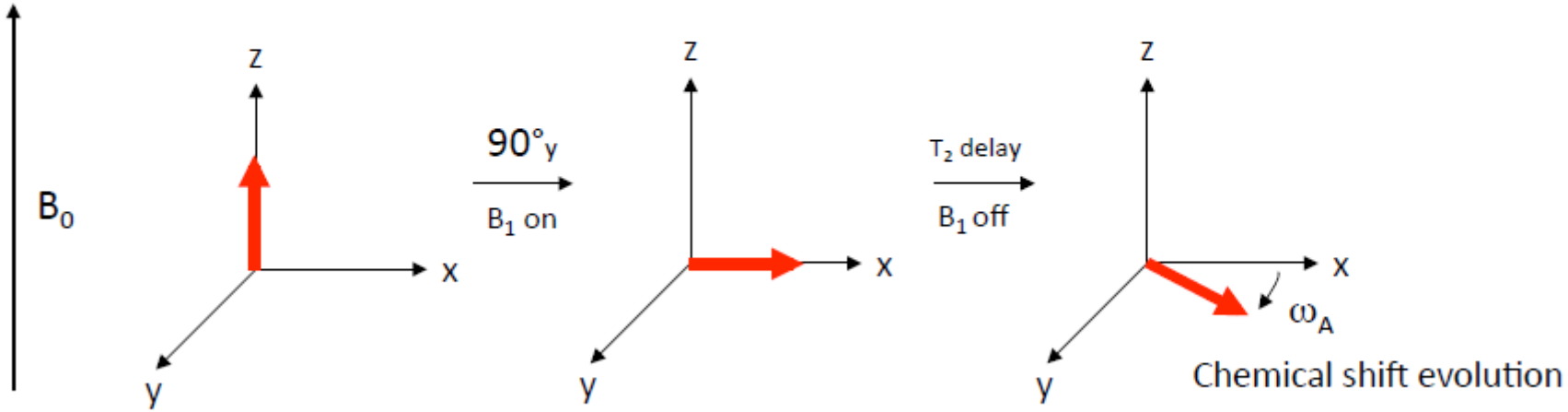


HSQC+ ZZ

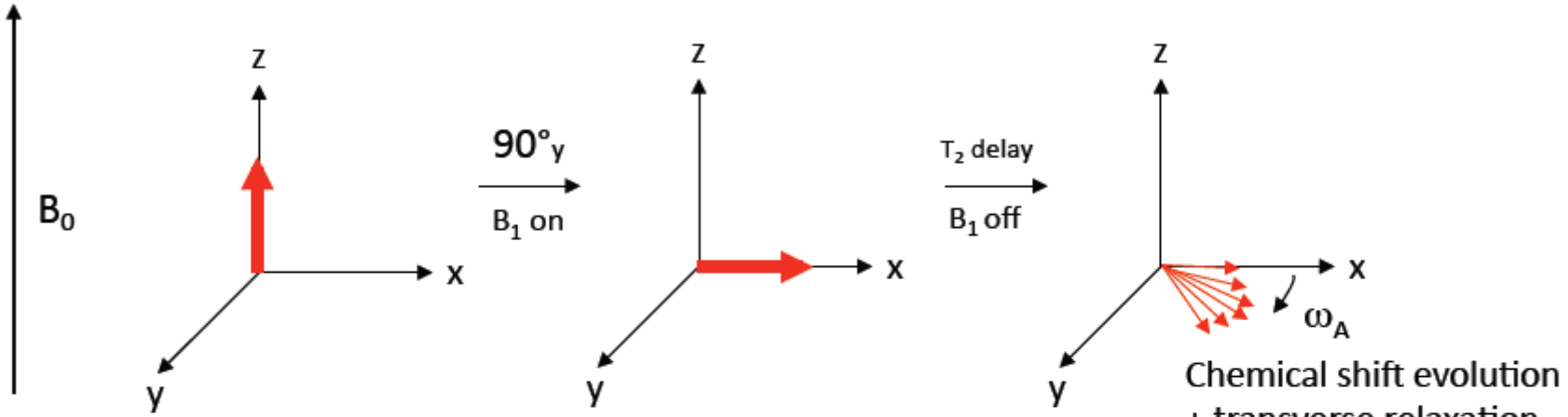


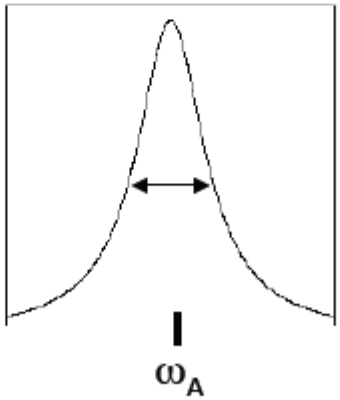
(If no exchange, no additional peaks will be observed)

CPMG: Transverse Relaxation & Conformational Exchange



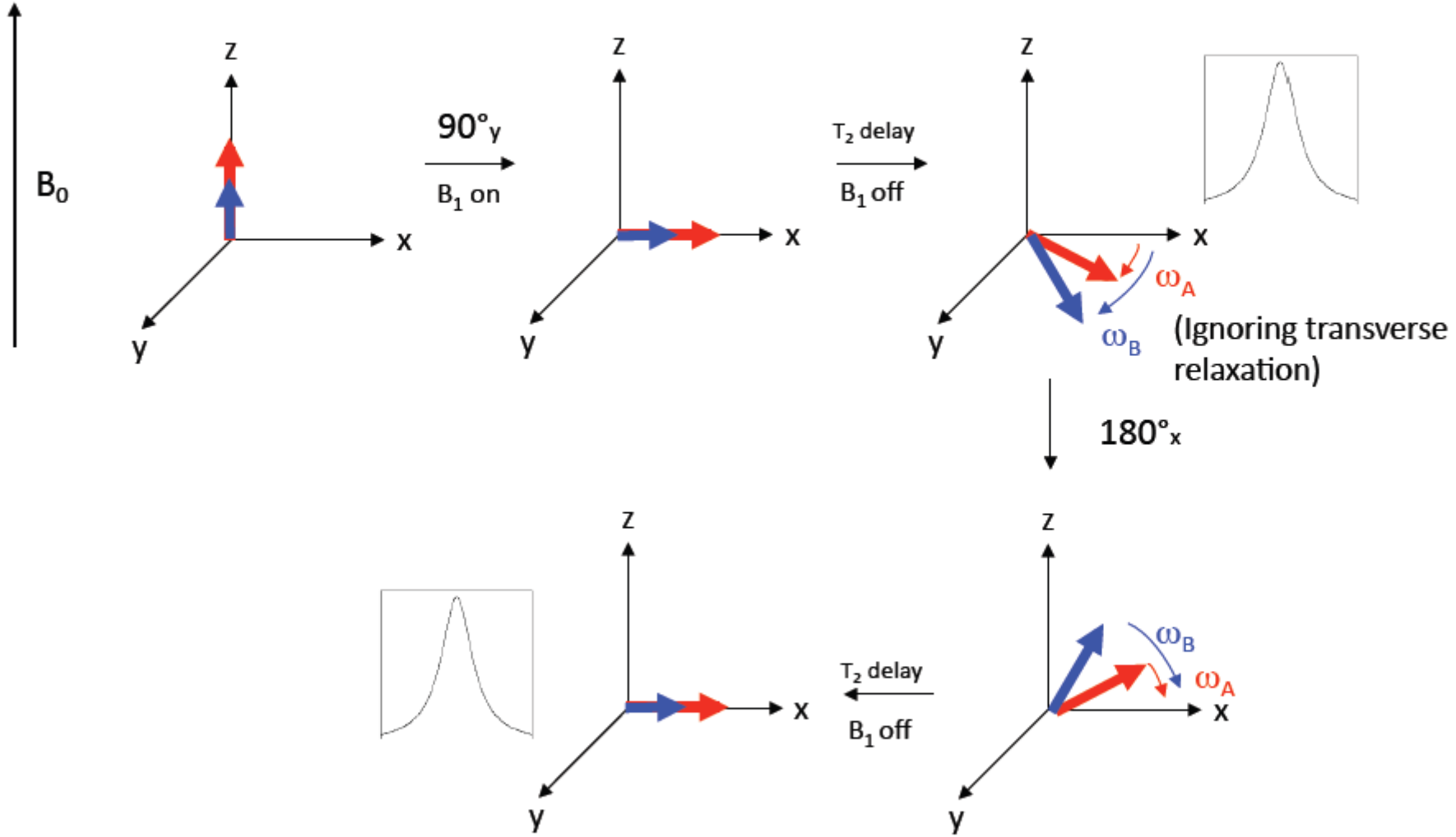
CPMG



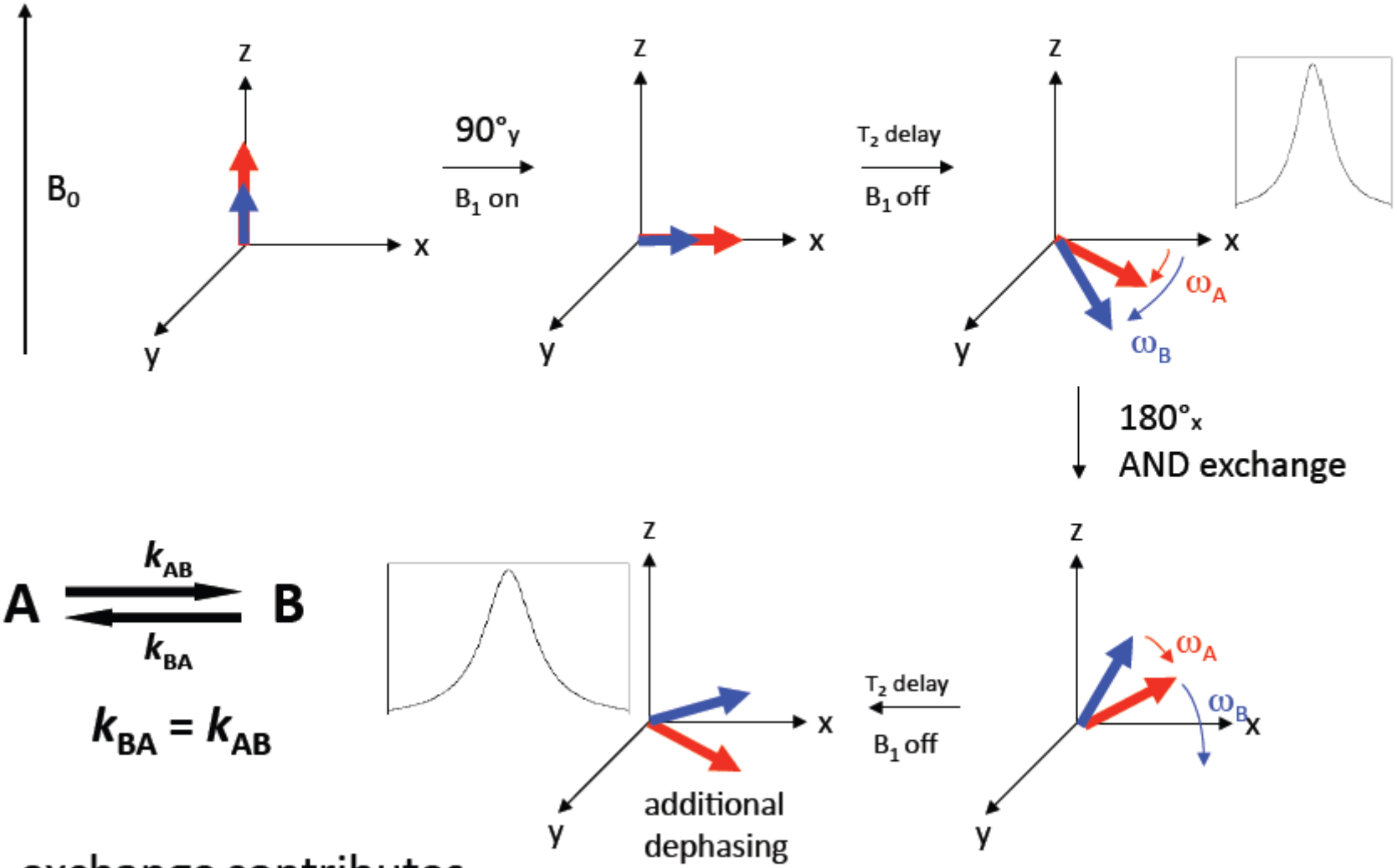


Intrinsic linewidth R_2^0

CPMG


B0
z
x
y
90°y
B1 on
z
x
y
T2 delay
B1 off
z
x
y
ωA
ωB
(Ignoring transverse relaxation)
180°x
z
x
y
T2 delay
B1 off
z
x
y
ωB
ωA
No exchange, spins refocused, same linewidth.

CPMG

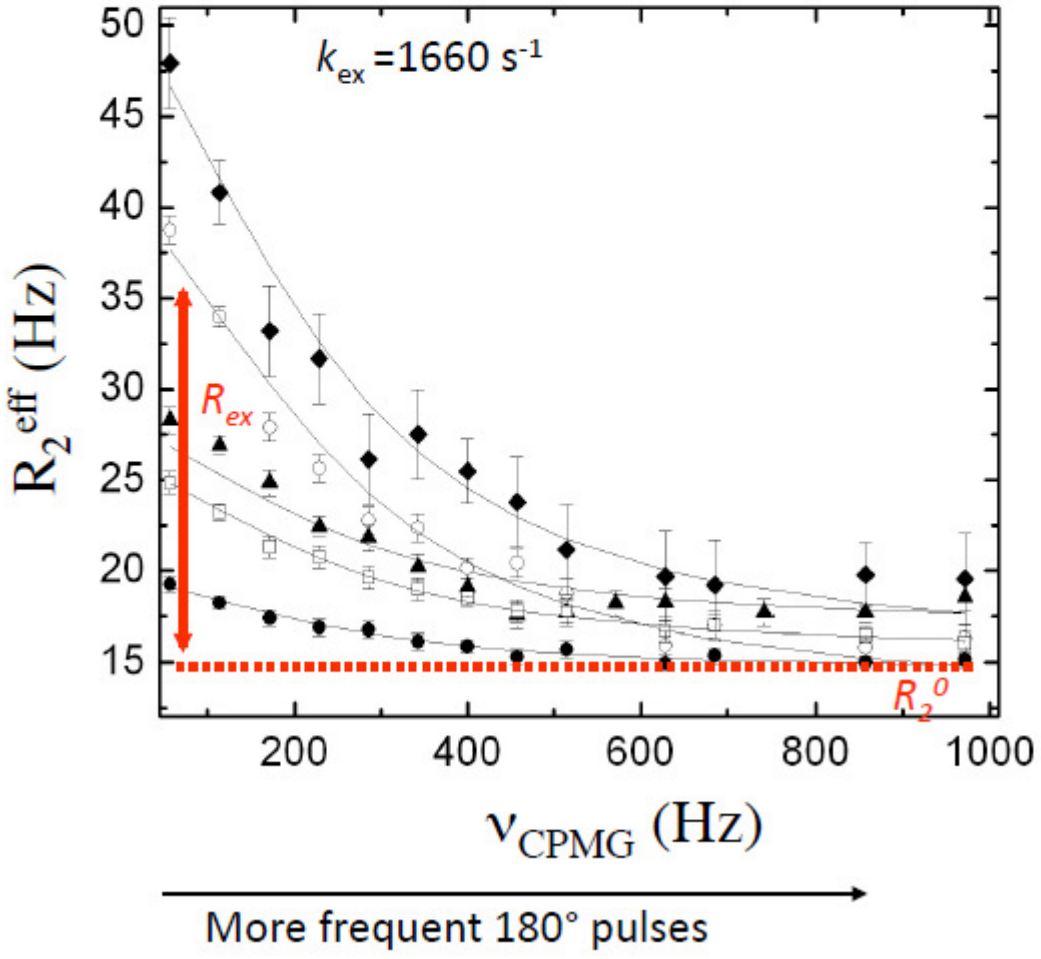


exchange contributes to line broadening

Note: need a chemical shift difference!

CPMG: Transverse Relaxation & Conformational Exchange

$$R_2(\nu_{CPMG}) = R_2^0 + (p_A p_B \Delta\omega^2/k_{ex})(1-(4\nu_{CPMG}/k_{ex})\tanh(k_{ex}/4\nu_{CPMG}))$$



$$R_{ex} = p_A p_B \Delta\omega^2/k_{ex}$$

What would a residue with no exchange look like?

Relate $\Delta\omega$ to known chemical shift differences to assign exchanging states

Use different field strength data to separate p_A and $\Delta\omega$.

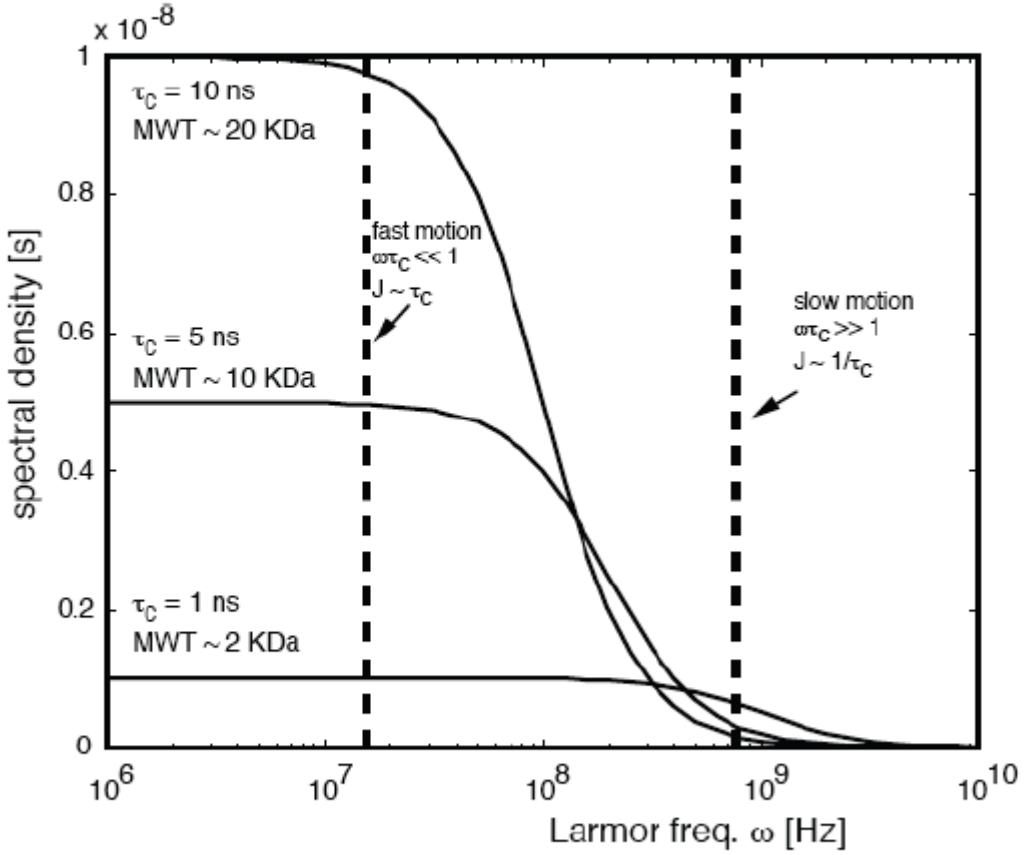
Fast Timescale Dynamics

- Less intuitive than slow timescale dynamics
- Measure ps-ns motions
  - Faster fluctuations
  - Small motions
  - Large number of states
- Reflect entropy of system, although exact calculation is not particularly helpful
  - Reflects re-orientational motion only, not affected by motions along an internuclear vector
  - Only observing a small subset of protein nuclei
  - Ignores rest of protein, water, etc…

Fast Timescale Dynamics

- Measure R_1, R_2, and heternuclear NOE
  - Molecular tumbling (global) and protein motion (local) cause fluctuations in local magnetic field that lead to relaxation.
- Correlation between macroscopic relaxation and microscopic fluctuations follows the fluctuation-dissipation theorem.
  - Anisotropic interactions (chemical shift anisotropy and dipolar coupling) depend on orientation of molecule in magnetic field.
  - As the protein moves, the local field due to these interactions fluctuates, this causes loss of coherence (T2 relaxation) and return to equilibrium (T1 relaxation).
  - Sometimes the field has the right frequency to cause spin flips. This leads to NOE.
  - Relaxation depends on the spectral density, $J(\omega)$ – the probability of field fluctuations of each frequency/energy within the thermal fluctuations of the molecue. Only certain frequencies cause energy transfer between spins or between spins and lattice

Spectral Density



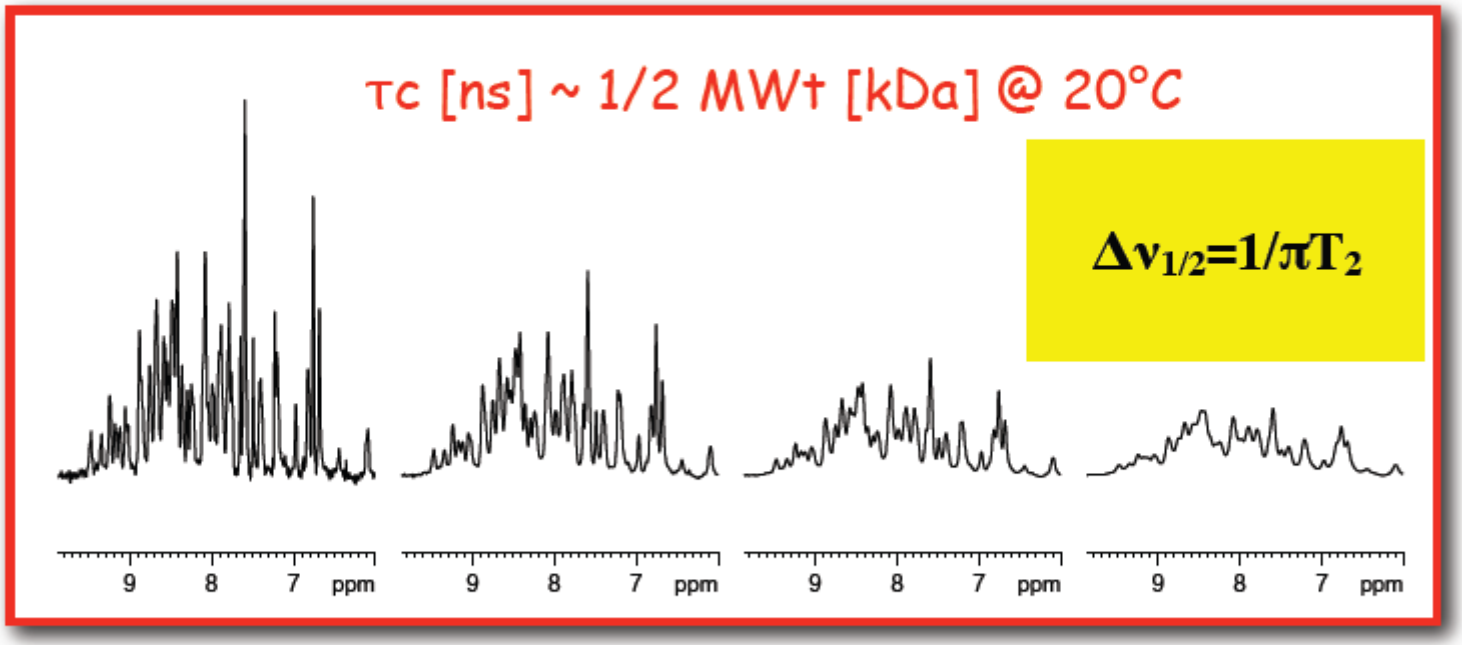
$$J(\omega) = \frac{2\tau_c}{1+(\omega\tau_c)^2}$$

Fig.: the spectral density or the efficiency of relaxation for different Larmor frequencies and rotational correlation times τ_c.

$$R_1 = 3AJ(\omega_N) + AJ(\omega_H - \omega_N) + 6AJ(\omega_H + \omega_N) + BJ(\omega_N)$$

$$\mathrm{NOE} = 1 + \frac{\sigma}{R_1}\frac{\gamma_H}{\gamma_N}$$

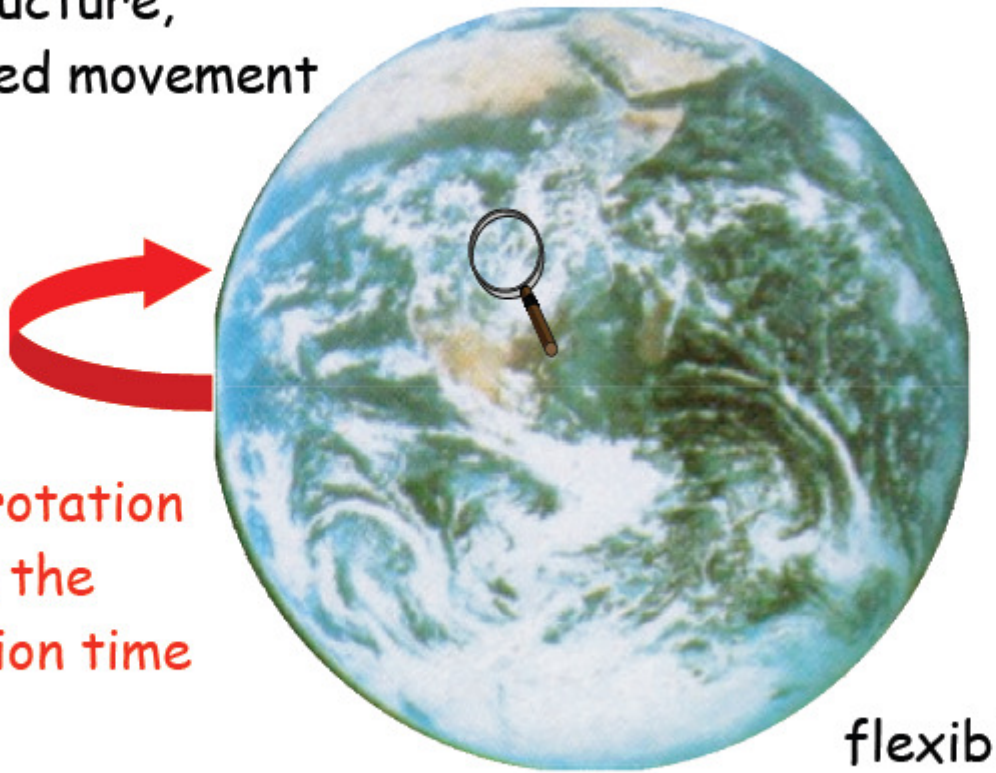
Efficient relaxation results in broad lines: The effect of increasing correlation times



τ_c	5ns	10ns	15ns	25ns
MW	10kDa	20kDa	30kDa	50kDa

Measurement of internal mobility

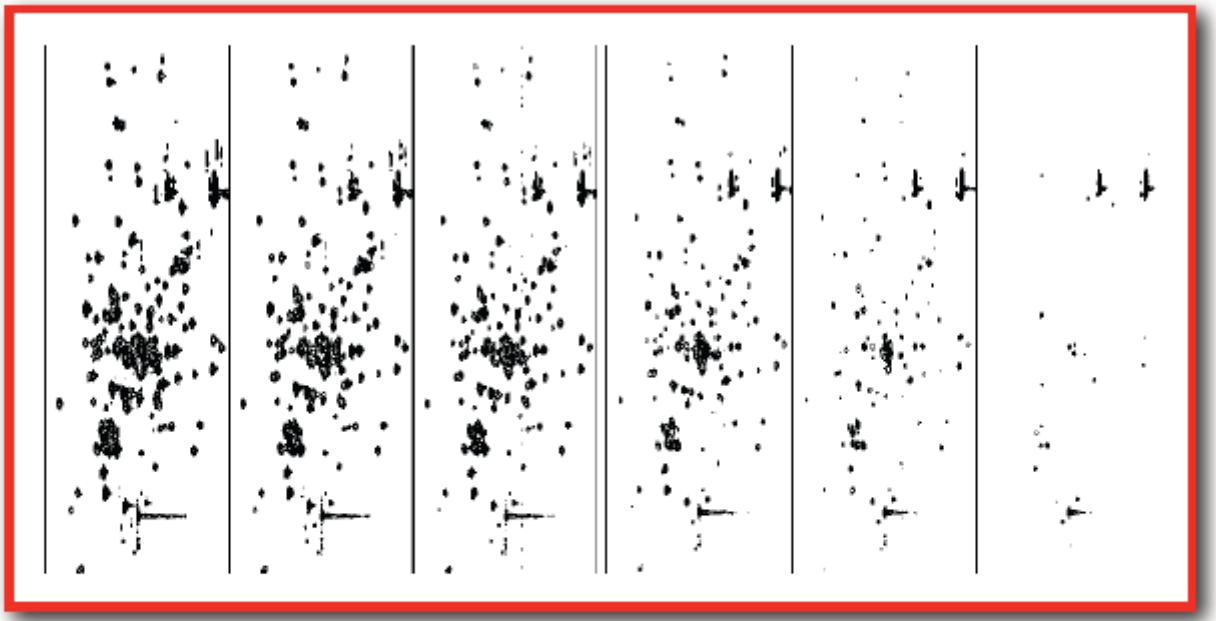
rigid structure,
correlated movement



overall rotation
with the
correlation time

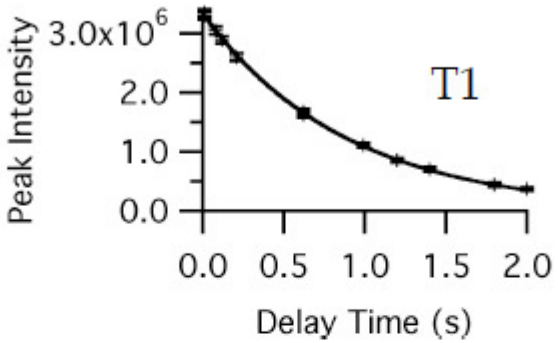
flexible parts:
additional uncorrelated
motion

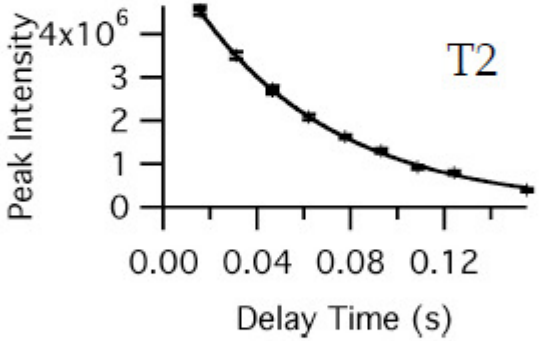
T1 series:

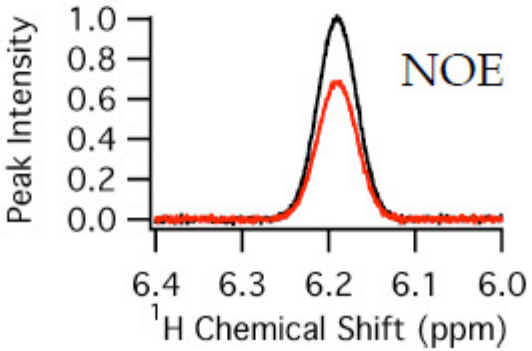


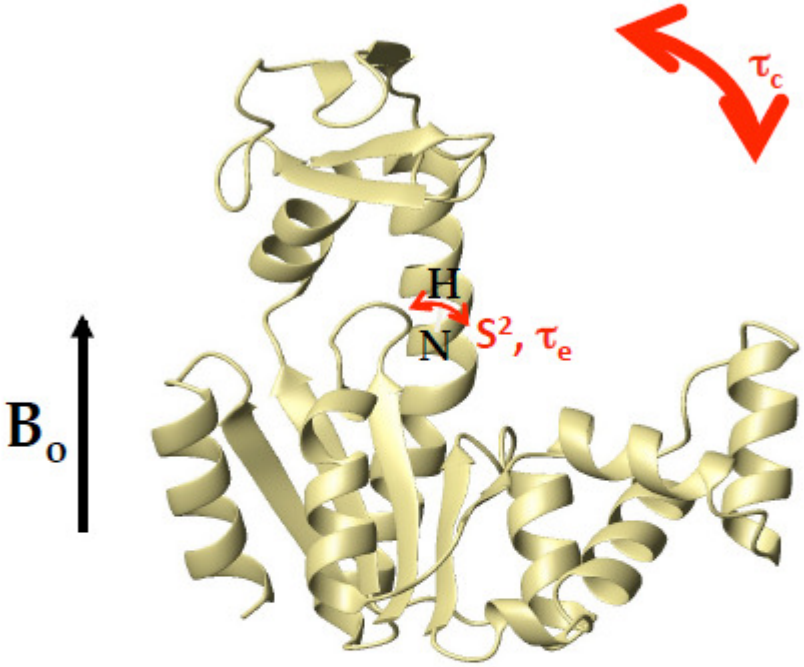
Record a series of ^{1}H, ^{15}N 2D spectra, in which the intensity of the peaks is modulated by relaxation

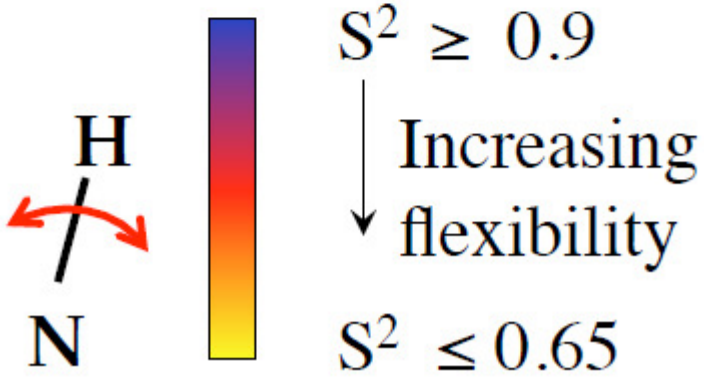
Fast timescale dynamics

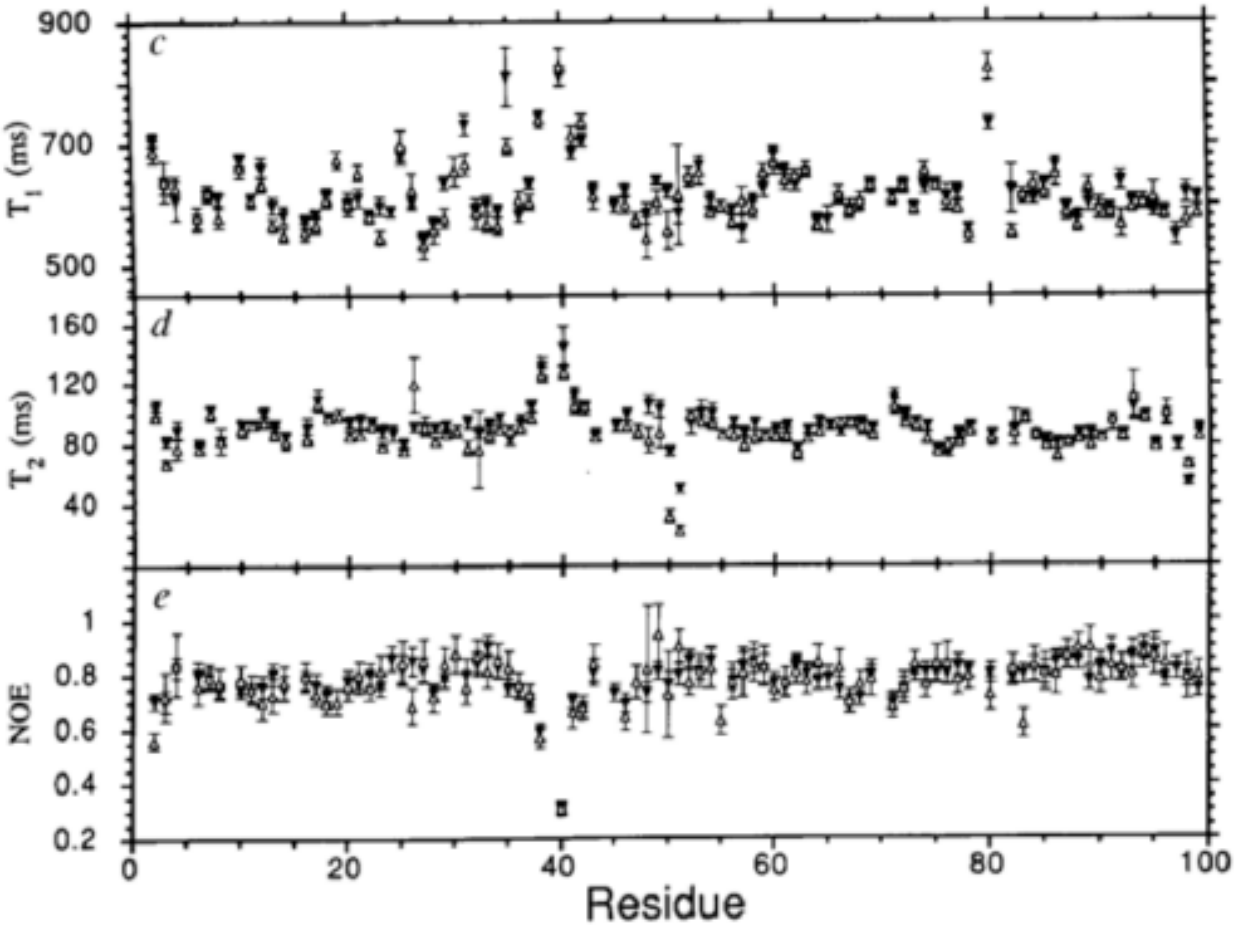






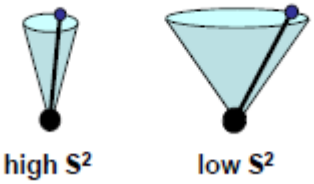




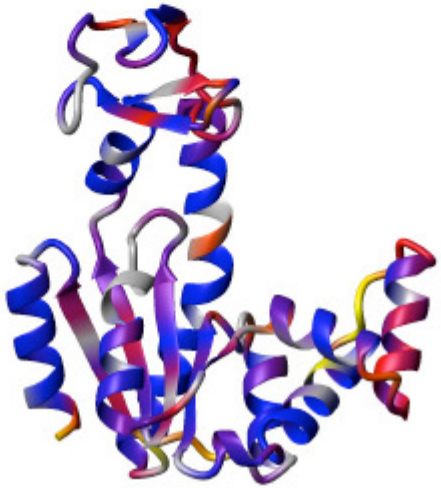

c
d
e
T_1 (ms)
T_2 (ms)
NOE
900
700
500
160
120
80
40
1
0.8
0.6
0.4
0.2
0
20
40
60
80
100
Residue

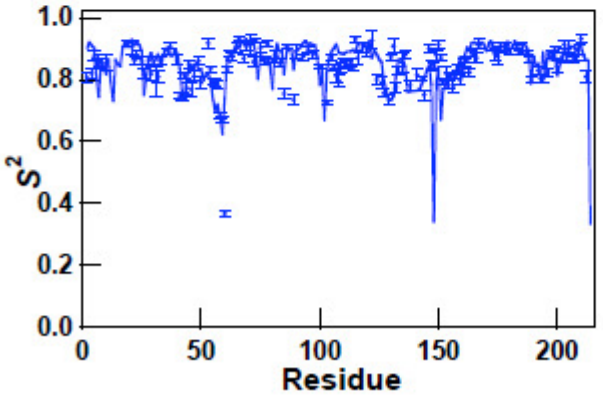
Fast time-scale dynamics

captures motion on ps-ns timescale



- Order parameters reflect fast timescale, small amplitude bond fluctuations
- Can calculate from MD trajectories
- Higher order parameters in regions of secondary structure
- Reflect local packing
  - can calculate order parameters reasonably well just by looking at local packing (# interactions around a given residue)
  - Reasonable agreement with B-factors from crystal structure, if regions affected by crystal packing between molecules are avoided.





Proteins are typically "stable" by only 5-10 kcal/mole

Compare to other bond energies:

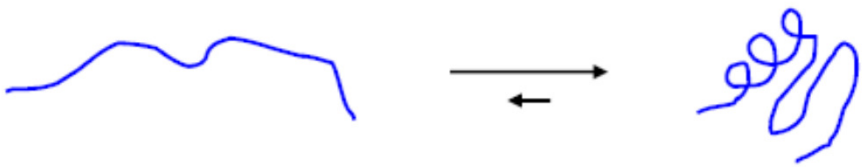
Bond Type	ΔG (kcal/mole)
hydrogen bond	1-3
ATP hydrolysis	~7
C-H covalent bond	~100

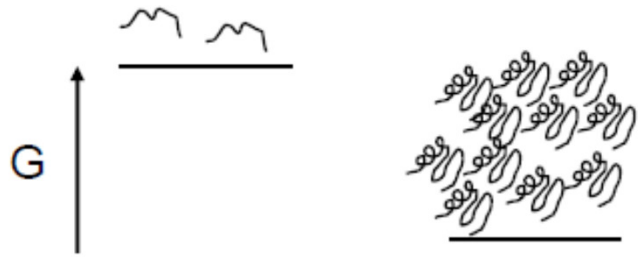
Protein folding stability is precariously balanced $\Delta G = \Delta H - T\Delta S$

enthalpically favored
entropically unfavored (or is it?)

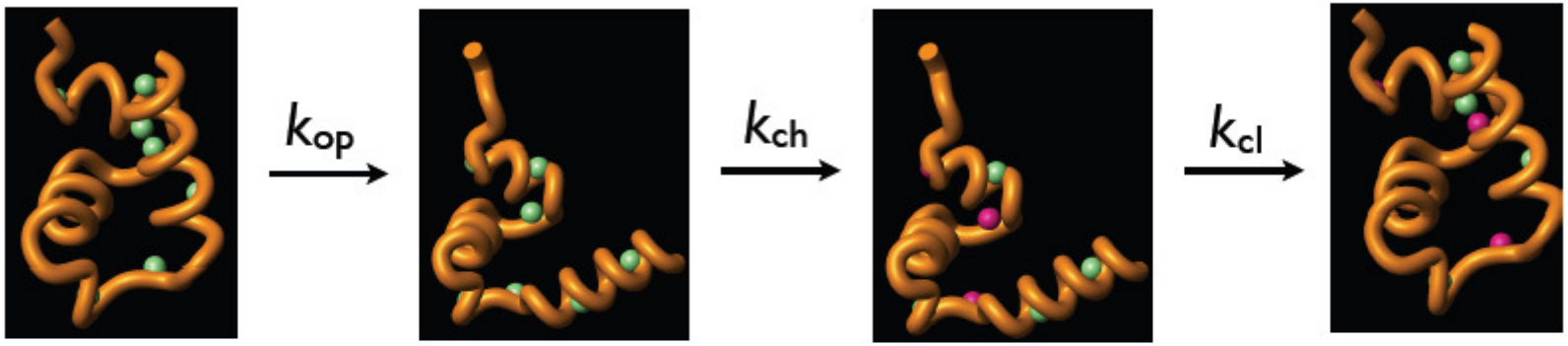
Proteins are in equilibrium with the denatured state.

Because the ΔG is ~5-10 kcal/mole, there is a small (but not insignificant) population of unfolded proteins.





Hydrogen/Deuterium (H/D) exchange



$$k_{obs}=k_{op}\ k_{ch}/(k_{op} + k_{cl} + k_{ch})$$

EX2: $k_{cl}>>k_{ch}$ $\qquad k_{obs}=k_{op}\ k_{ch}/(k_{cl}) = K_{op}\ k_{ch}$

K_{op} is referred to as the protection factor, P

$$\Delta G_{op} = -RT\ln K_{op}$$

Hydrogen/Deuterium (H/D) exchange