Using NMR to study Macromolecular Interactions

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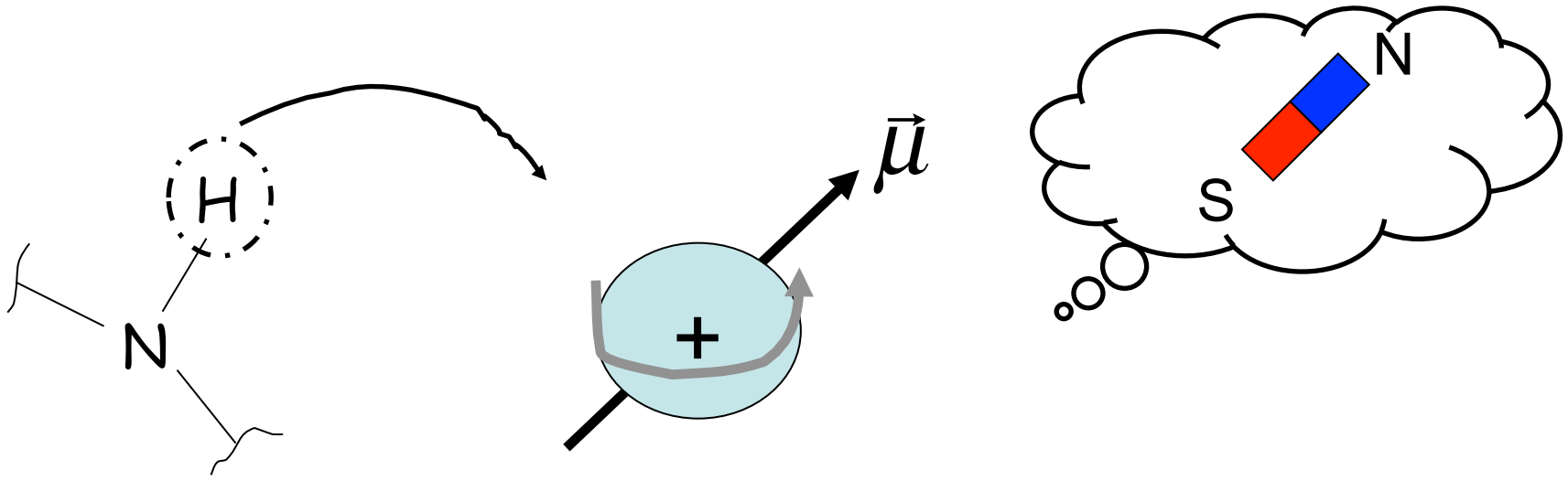
Outline

- Review of basic NMR experiment
- Multidimensional NMR
- Monitoring ligand binding
- Structure Determination

Review: Nuclear Spins are Microscopic Bar Magnets



Protein Fragment Magnetic Moment Bar Magnet

Magnetic moment $\vec{\mu} = \gamma \vec{S}$ Angular Momentum

The proportionality constant γ: strength of bar magnet

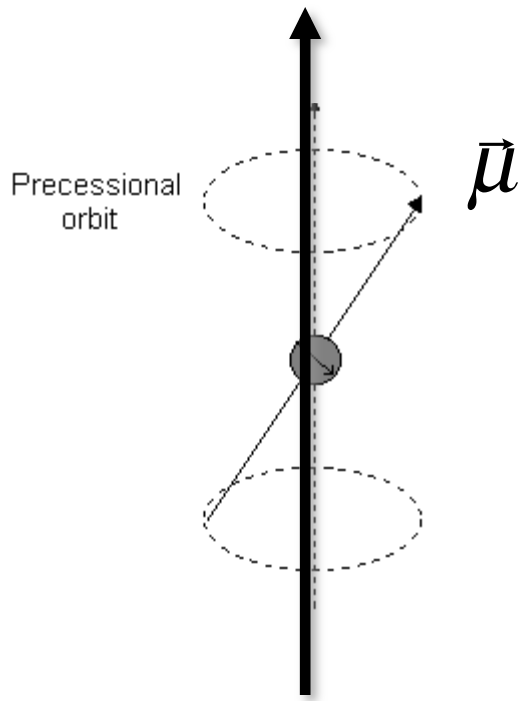
Equation of Motion

$$\frac{d\vec{\mu}}{dt} = \gamma \vec{B} \text{ x } \vec{\mu}$$

Based on magnetic torque:

$$\frac{d\vec{L}}{dt} = \vec{B} \times \vec{L}$$

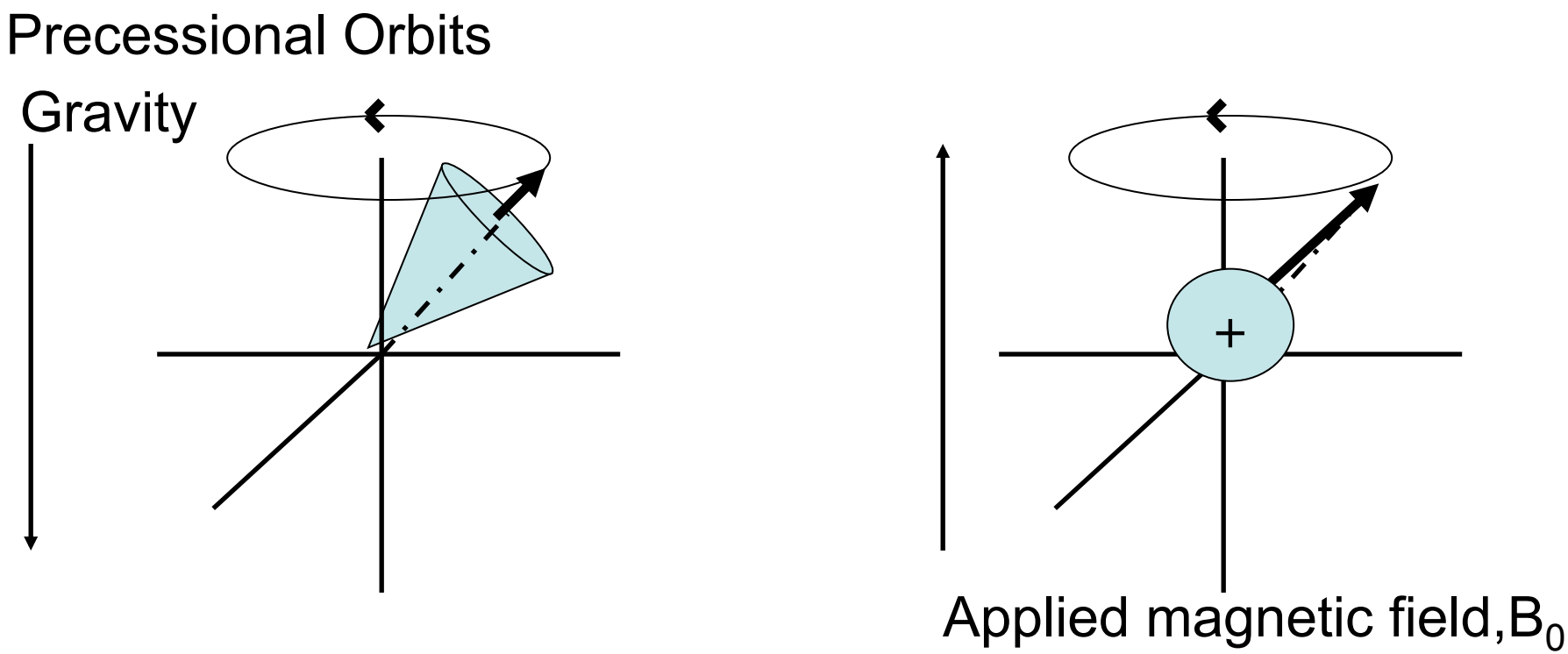
Spin Precession



Magnetic Field, Bo

Precession frequency: $\gamma B_0 = \omega_0$

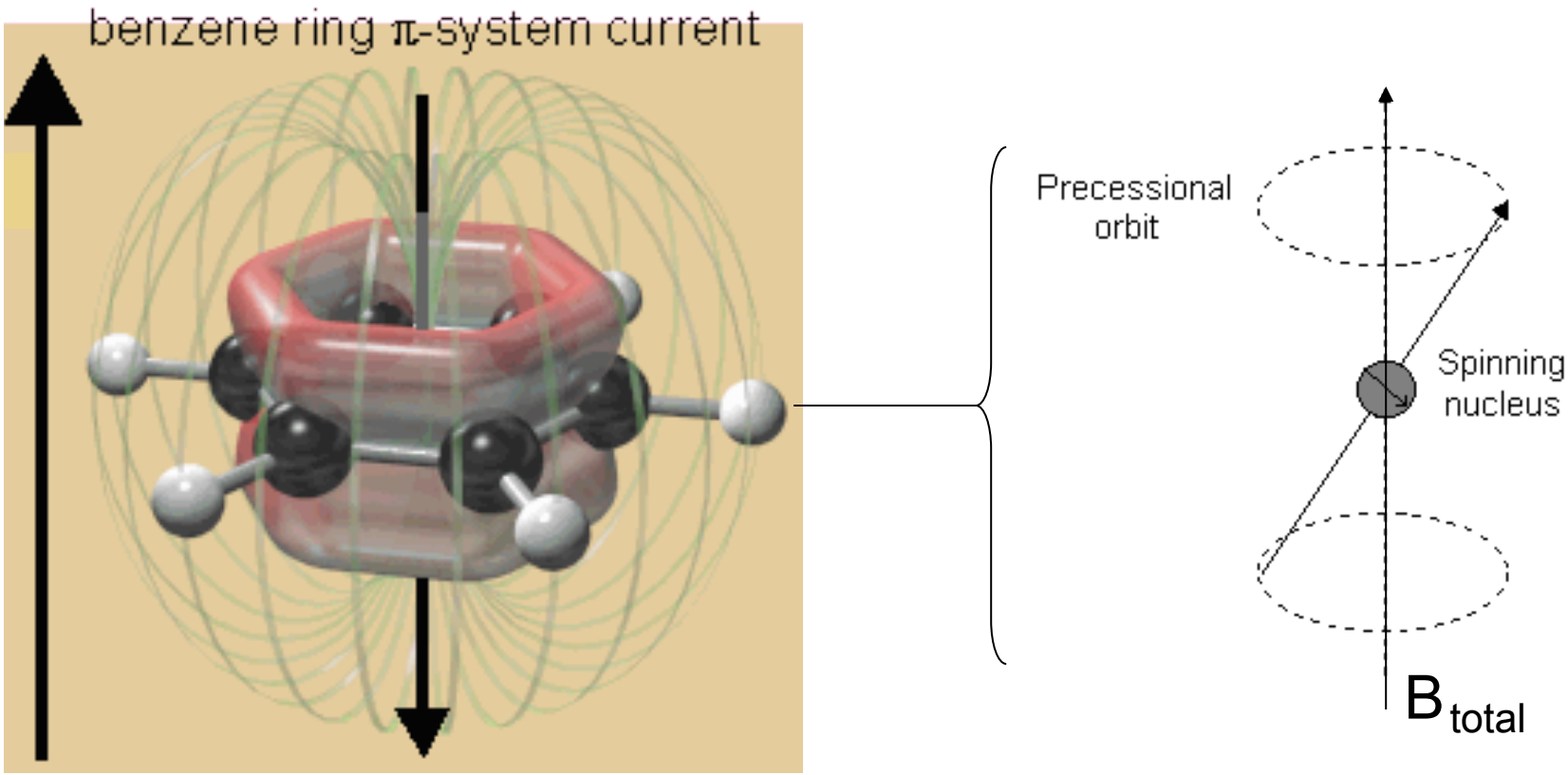
Driving Forces for Precession



Spinning Top

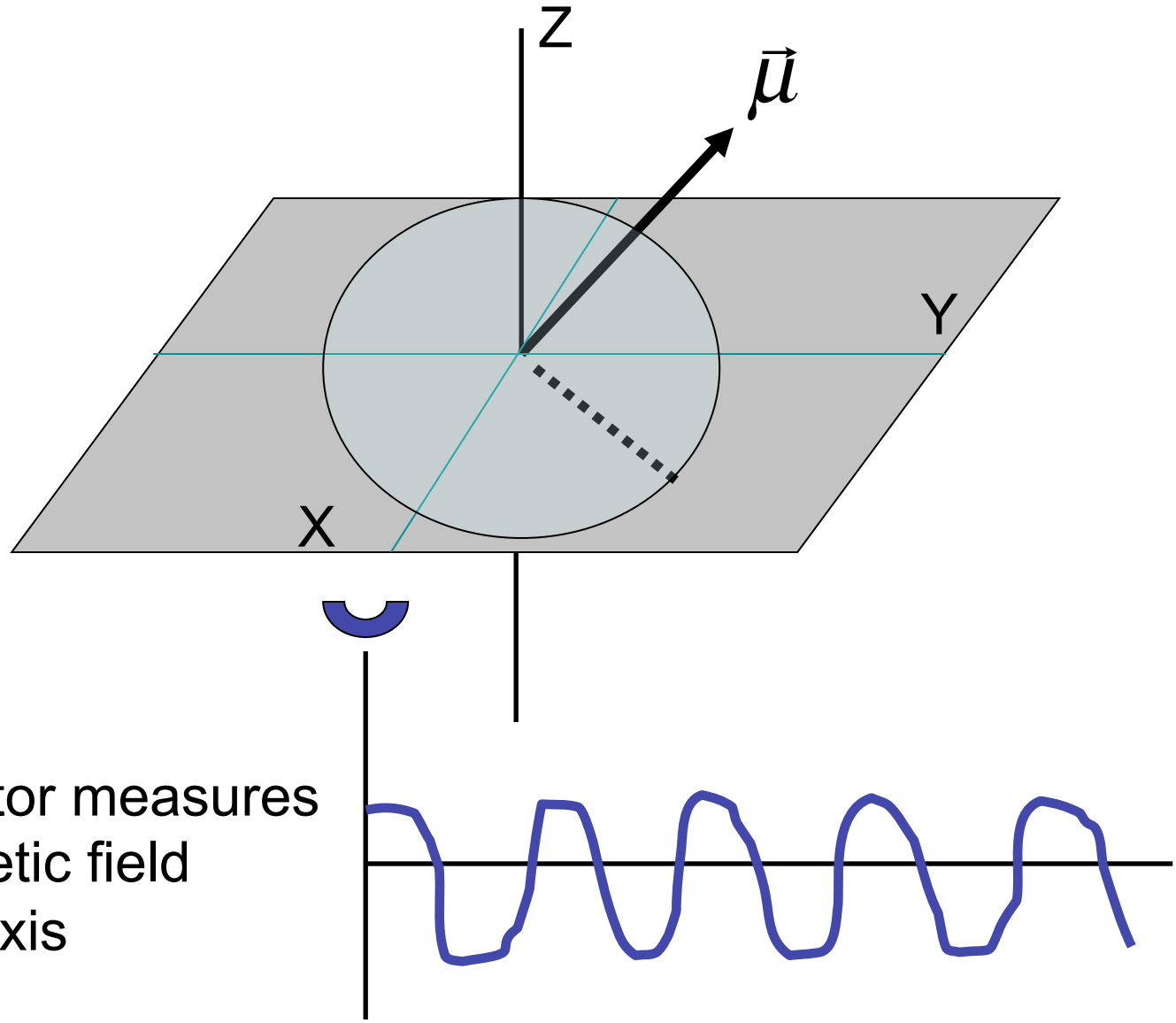
Spinning Nucleus

Nuclear Spins Report Local Environment



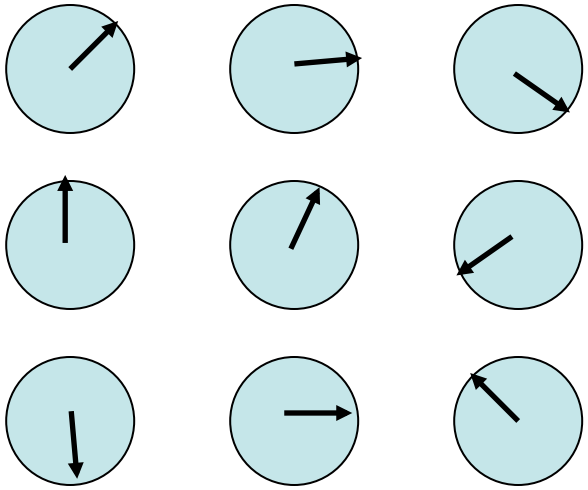
$B_{applied}$ + B_{local} = B_{total} **determines precession**

Detection of Spin Precession



Detector measures magnetic field on x-axis

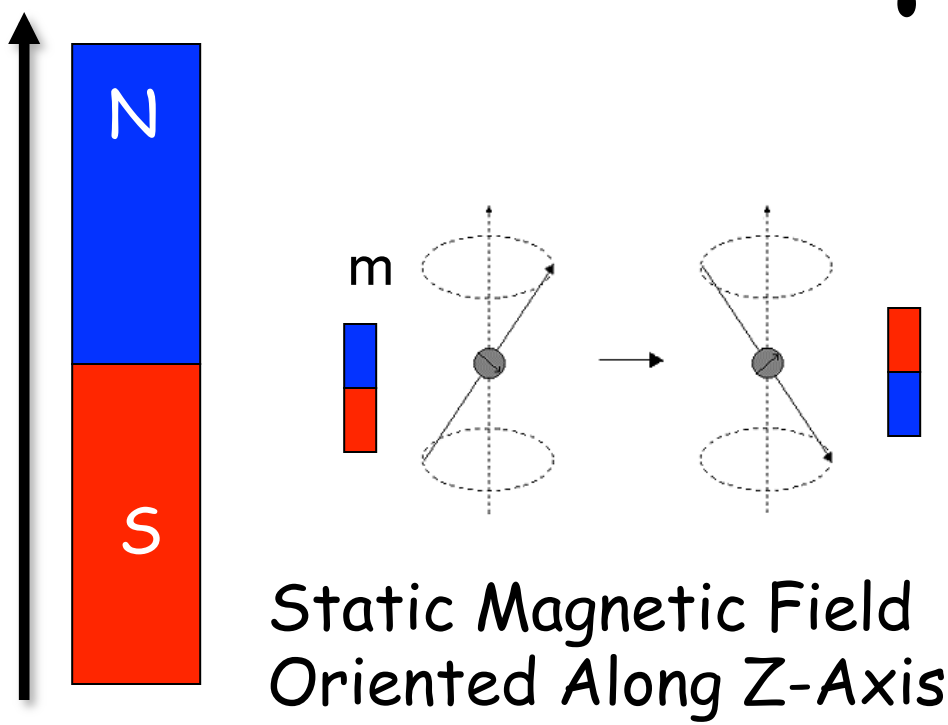
Net Magnetization



$$\left.\begin{aligned} \mathrm{M_x} &= \sum_j \mu_{\mathrm{x}}^j = 0 \\ \mathrm{M_y} &= \sum_j \mu_y^j = 0 \end{aligned}\right\} \text{No Transverse Magnetization at equilibrium}$$

Magnetic Energy

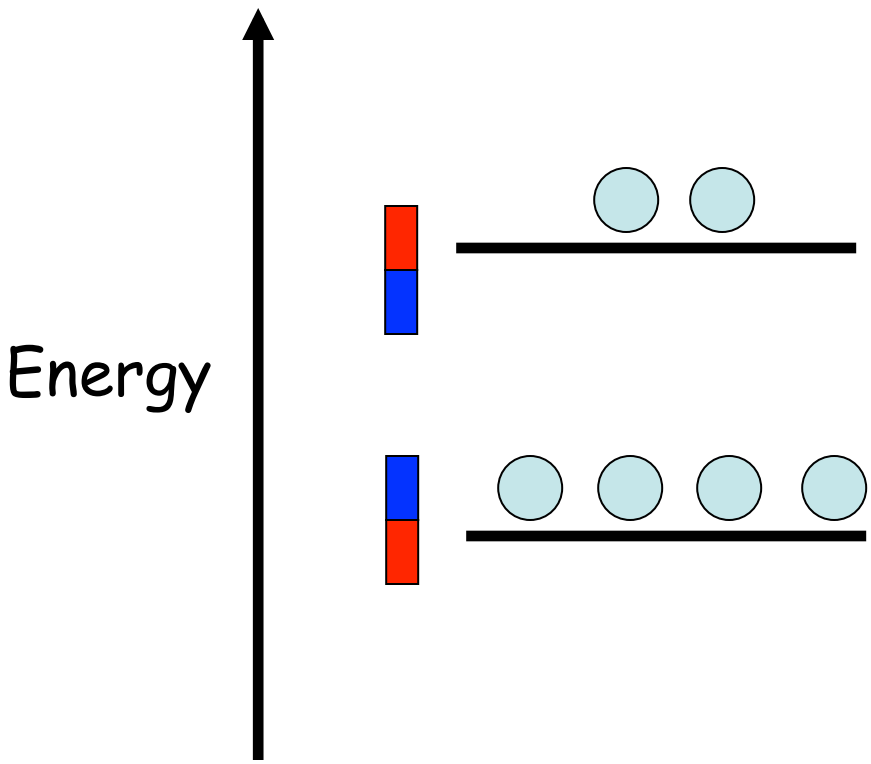
$$E = -\vec{\mu} \bullet \vec{B}$$



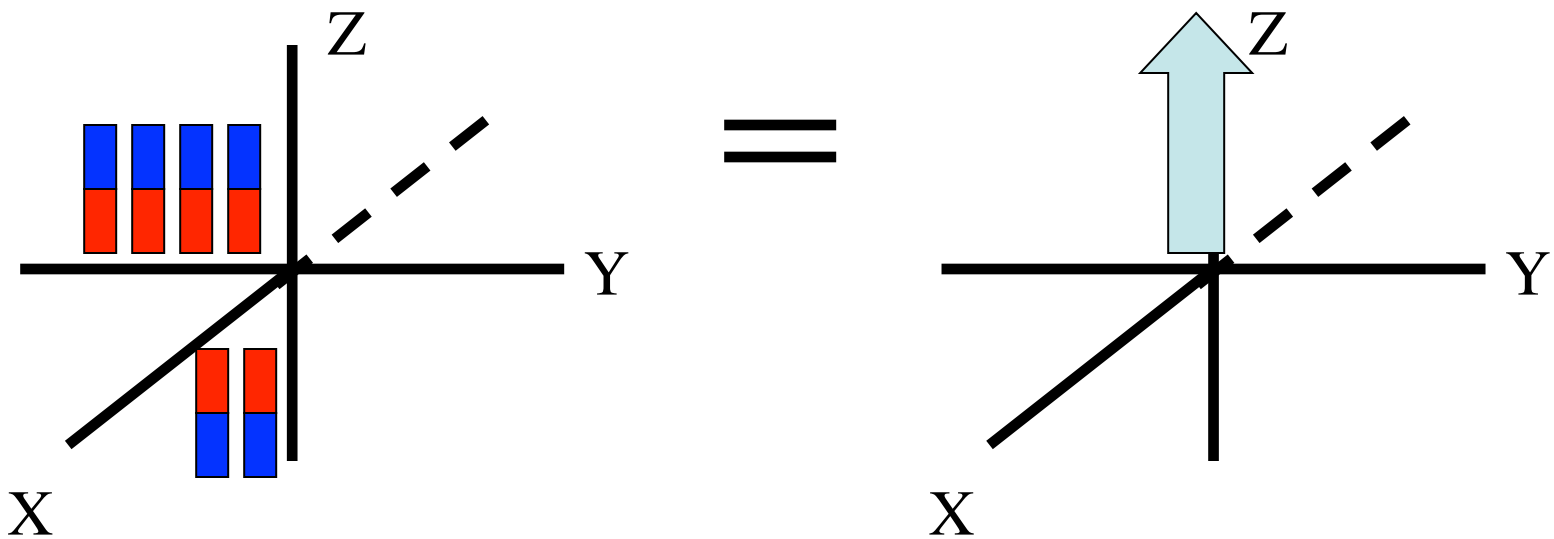
Static Magnetic Field
Oriented Along Z-Axis

$$E = -\mu_z B_z$$

Energy States (spin-1/2 nucleus)

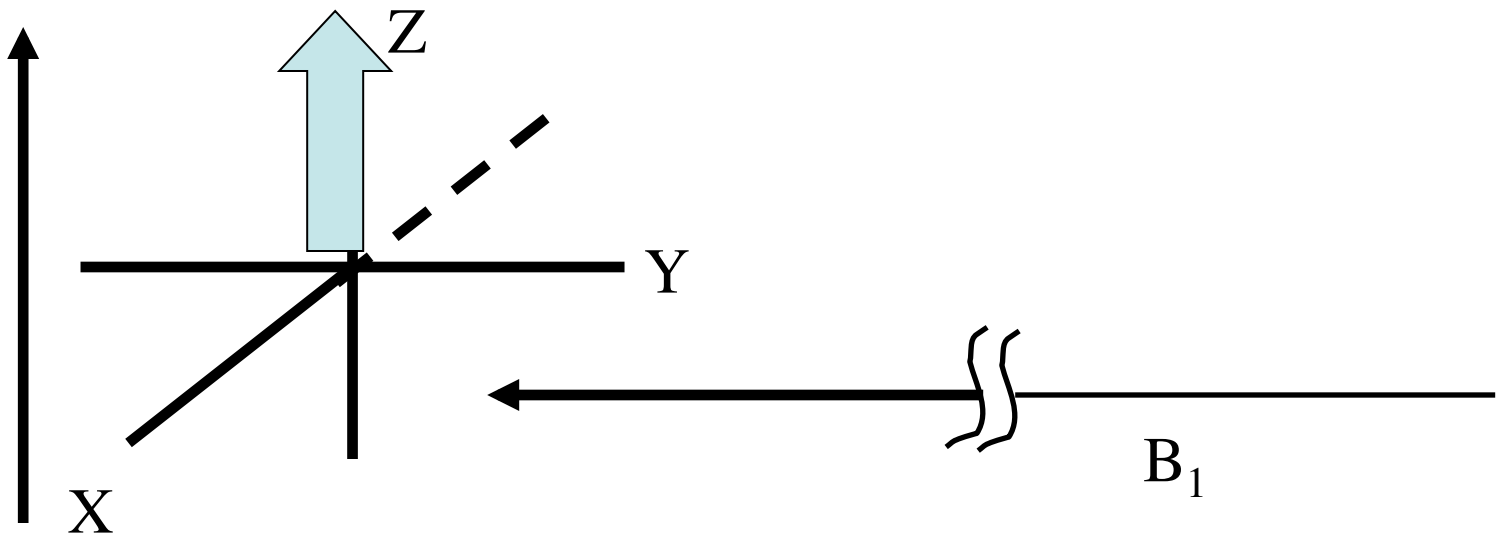


Net Magnetization along Z Axis



$$\sum_j \mu_z^j = M_z$$

Thought experiment: apply 2nd field along Y Axis

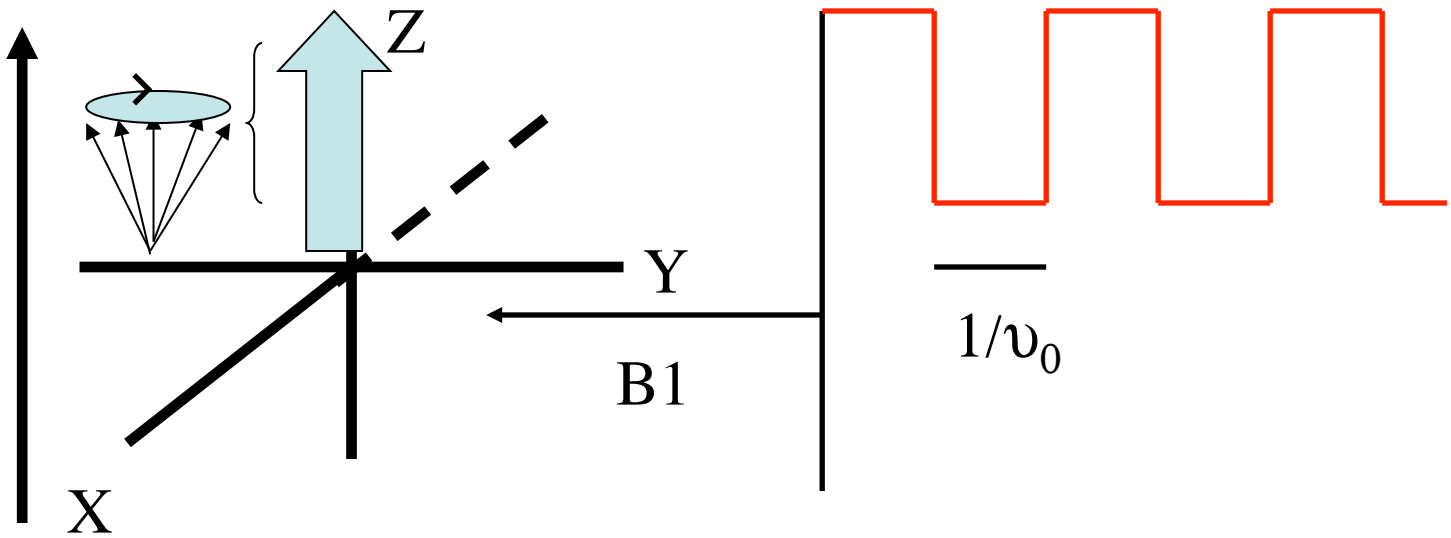


Bo

If $B_1 >> B_0$, M_Z would rotate about B_1.
Leave B_1 on until X axis reached ----> transverse magnetization
Approach is not practical.

Same effect achieved with weak, resonant oscillating field

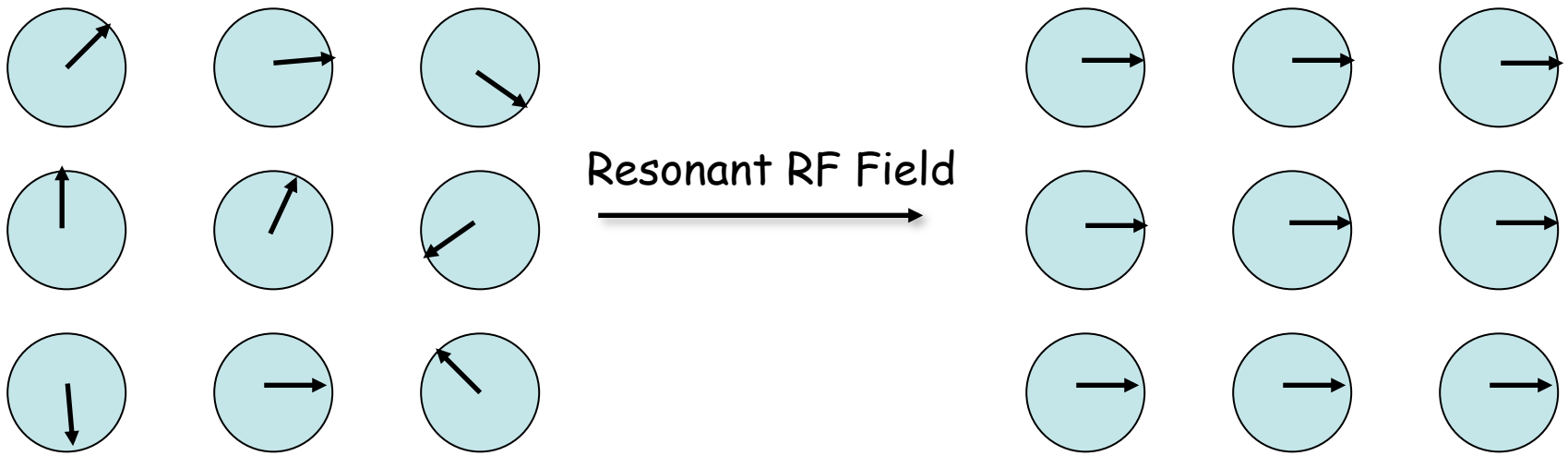
Bo



Turn B_1 on and off with a frequency matching the precessional frequency

Resonance

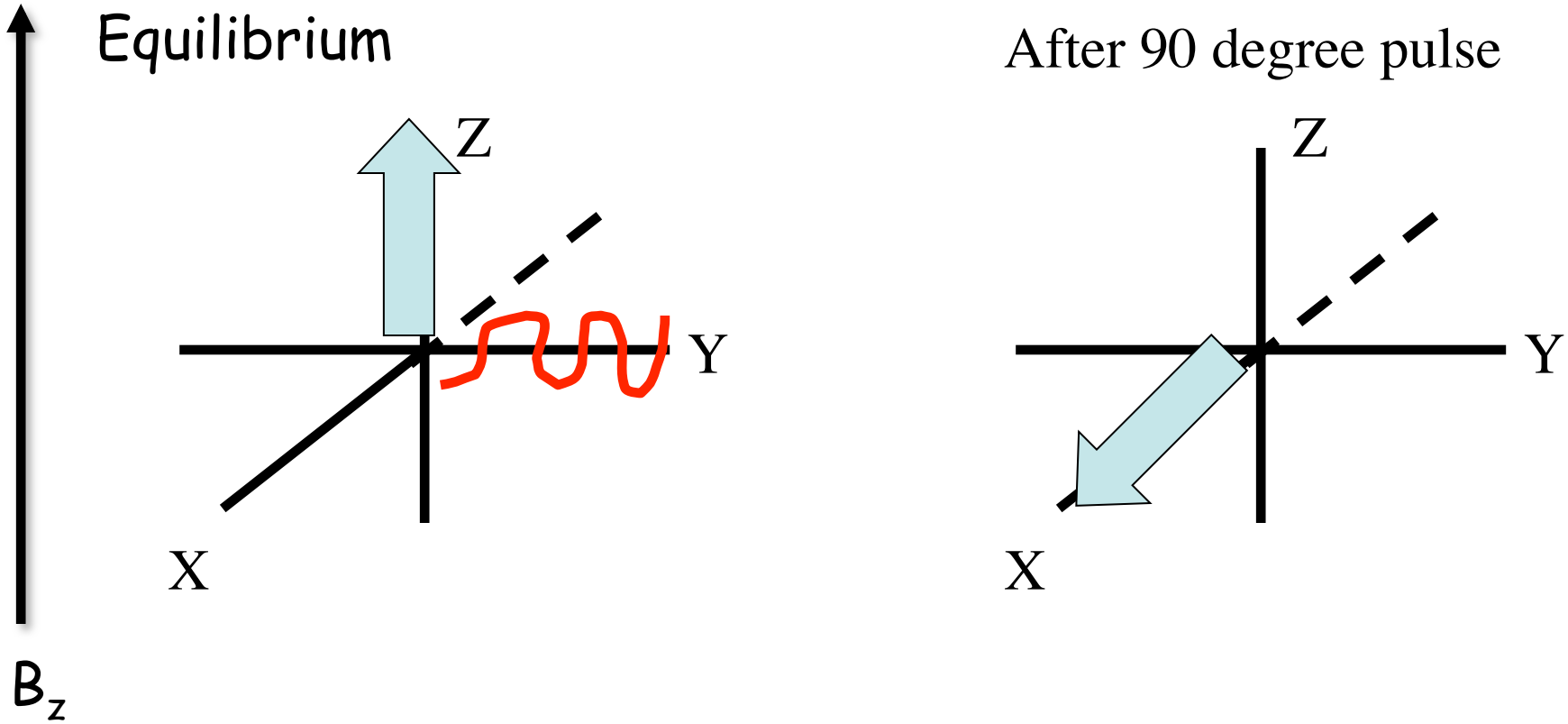
Ensemble of Nuclear Spins



Random Phase
No NMR Signal

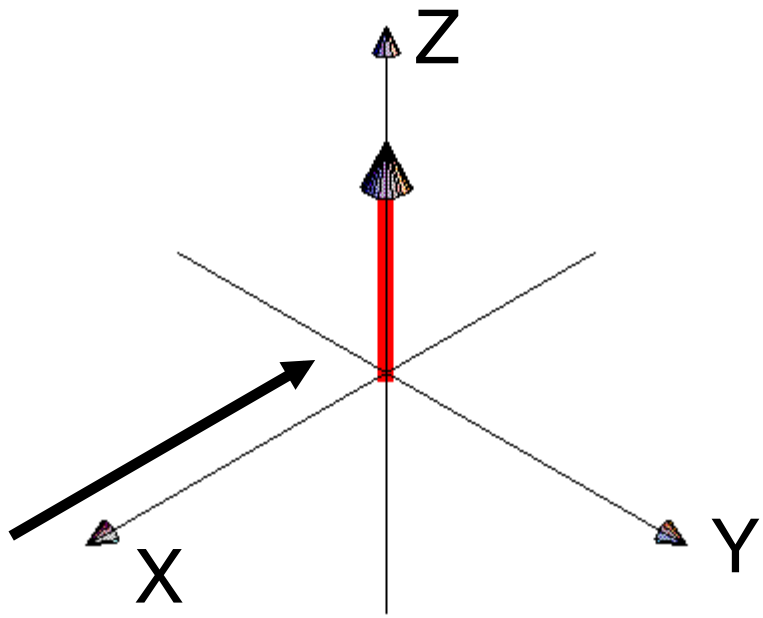
Phase Synchronization
NMR Signal!

Magnetization Vector Model



90y: Resonant 90 Degree Pulse

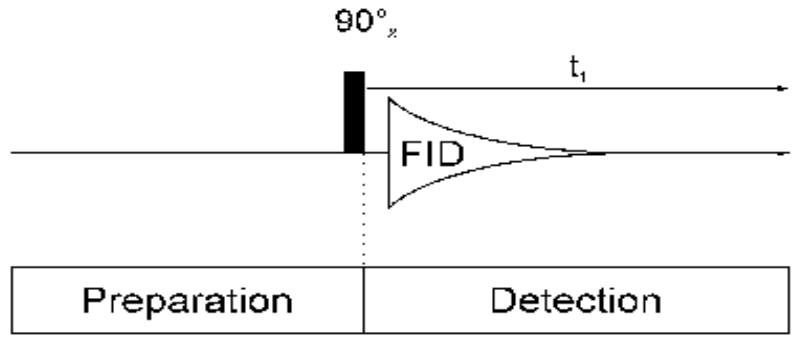
Resonant Pulse in Real Time

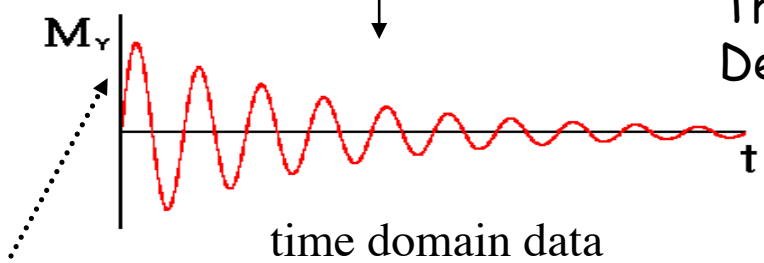


R.F. Field (applied at precession frequency)

Net magnetization rotated into transverse plane
Rotates due to static and local fields

Summary of 1D Experiment

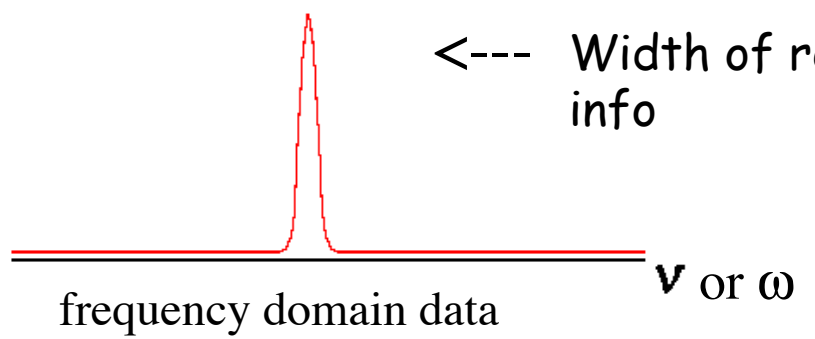




Transverse Magnetization
Decay constant T2

Amplitude propotional
to amount magnetization
prior to pulse

Fourier Transform (FT)

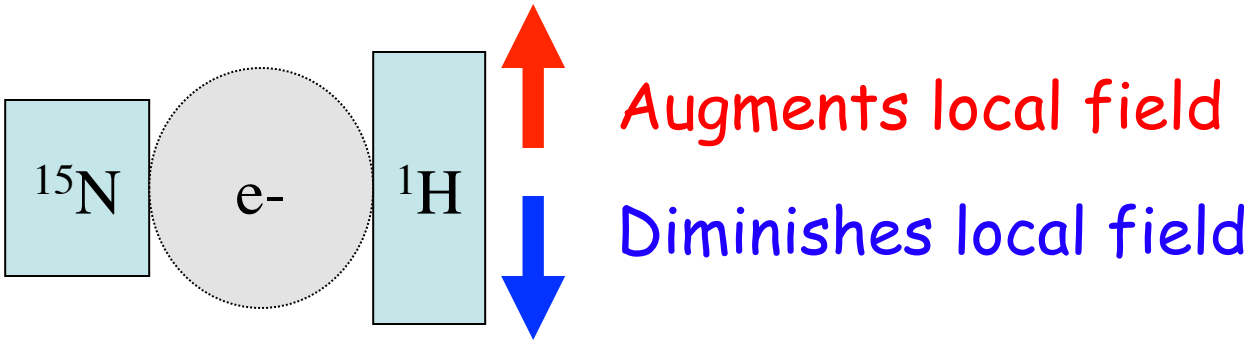


<--- Width of resonance--dynamical
info

Position of resonance --->
local magnetic environment

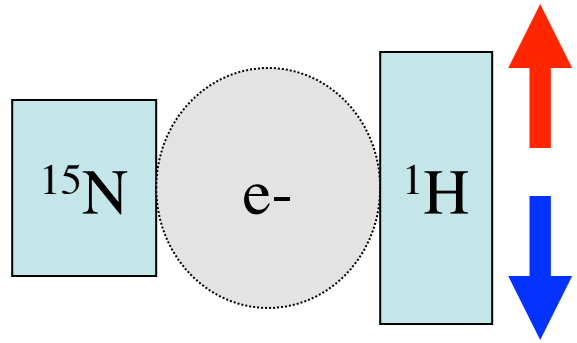
The J Coupling

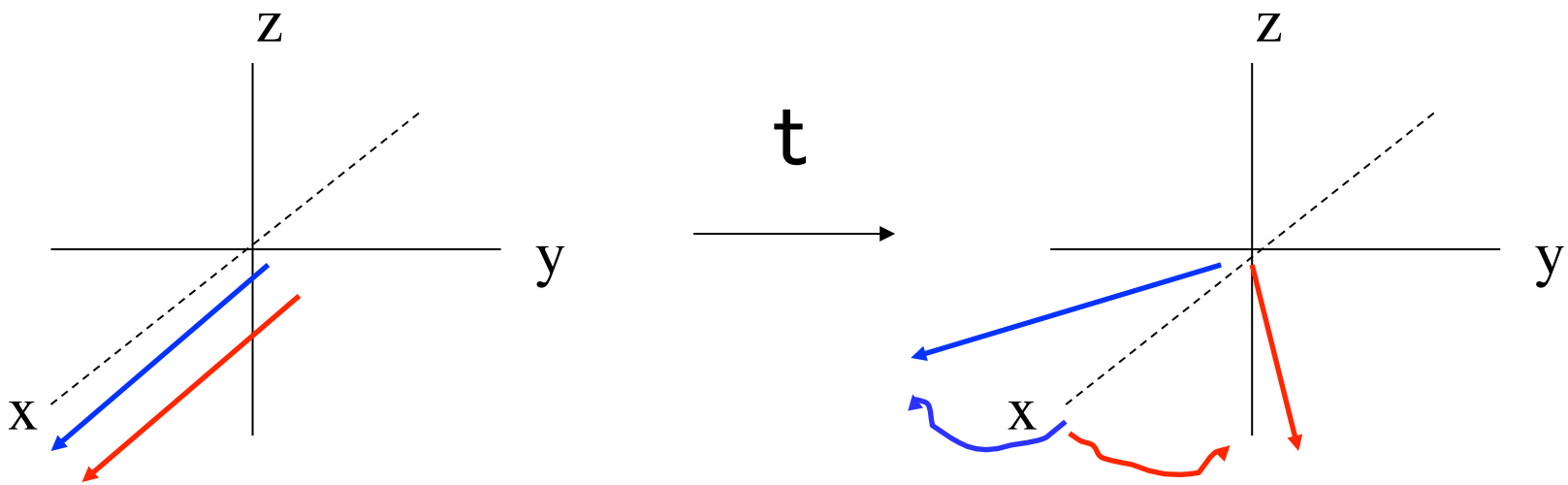
Consider two spin-1/2 nuclei (ie, ^{1}H and ^{15}N):



Effect transmitted through electrons in intervening bonds

Vector View

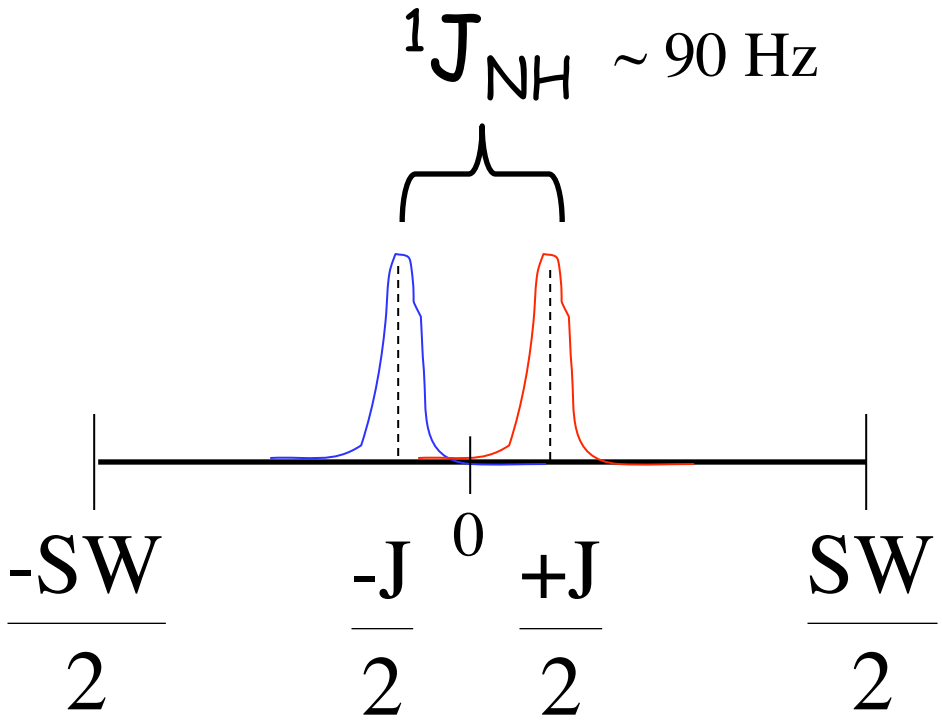




(After 90y pulse)

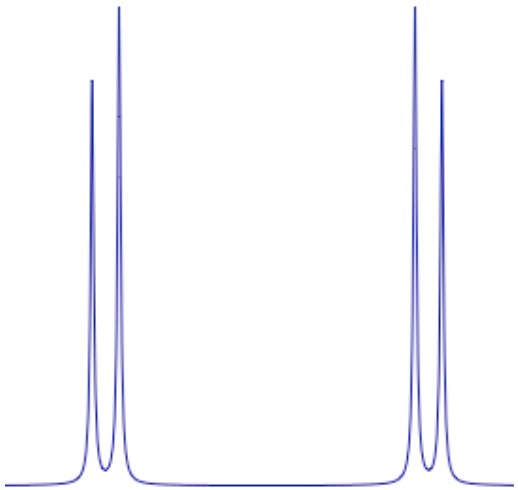
Components rotate faster or slower than rotating frame by +- J/2

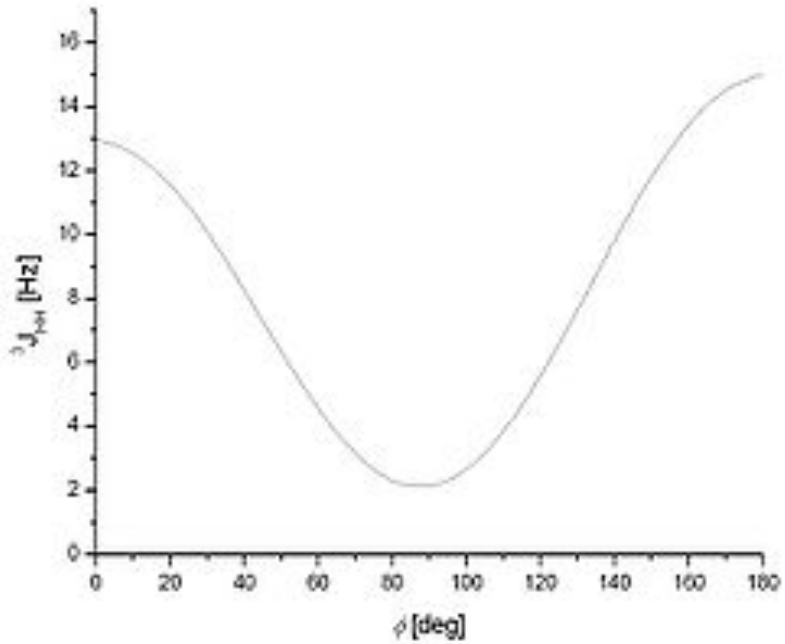
Spectrum with J coupling



^{15}N Detected Spectrum

J couplings contain information on structure





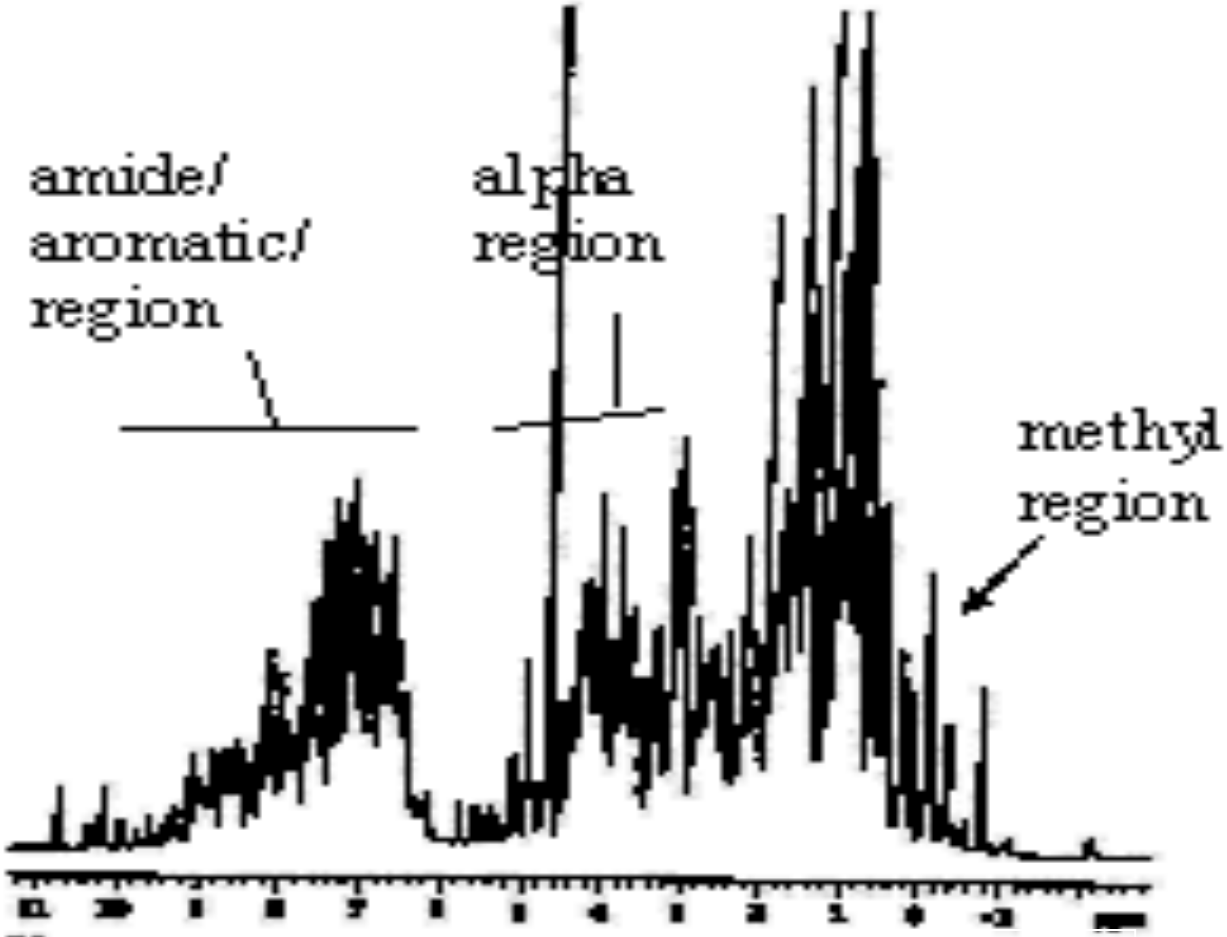
$$J(\phi) = A\cos^2\phi + B\cos\phi + C$$

Important Observables

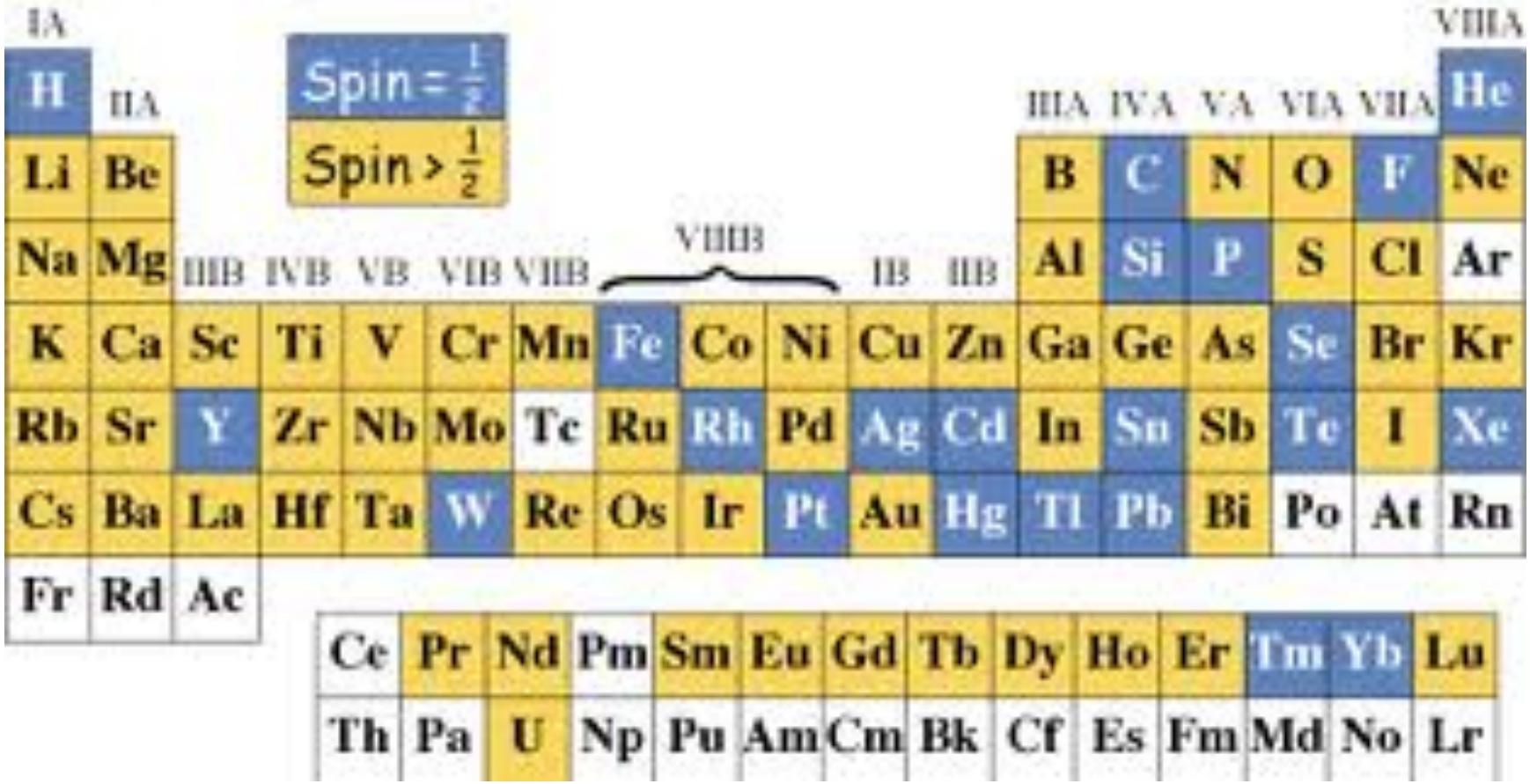
Chemical shift is a reporter of magnetic environment

The J coupling can inform torsion angles

Protein NMR Spectroscopy



Periodic Table of NMR active Nuclei



Isotopic Labeling Proteins for NMR

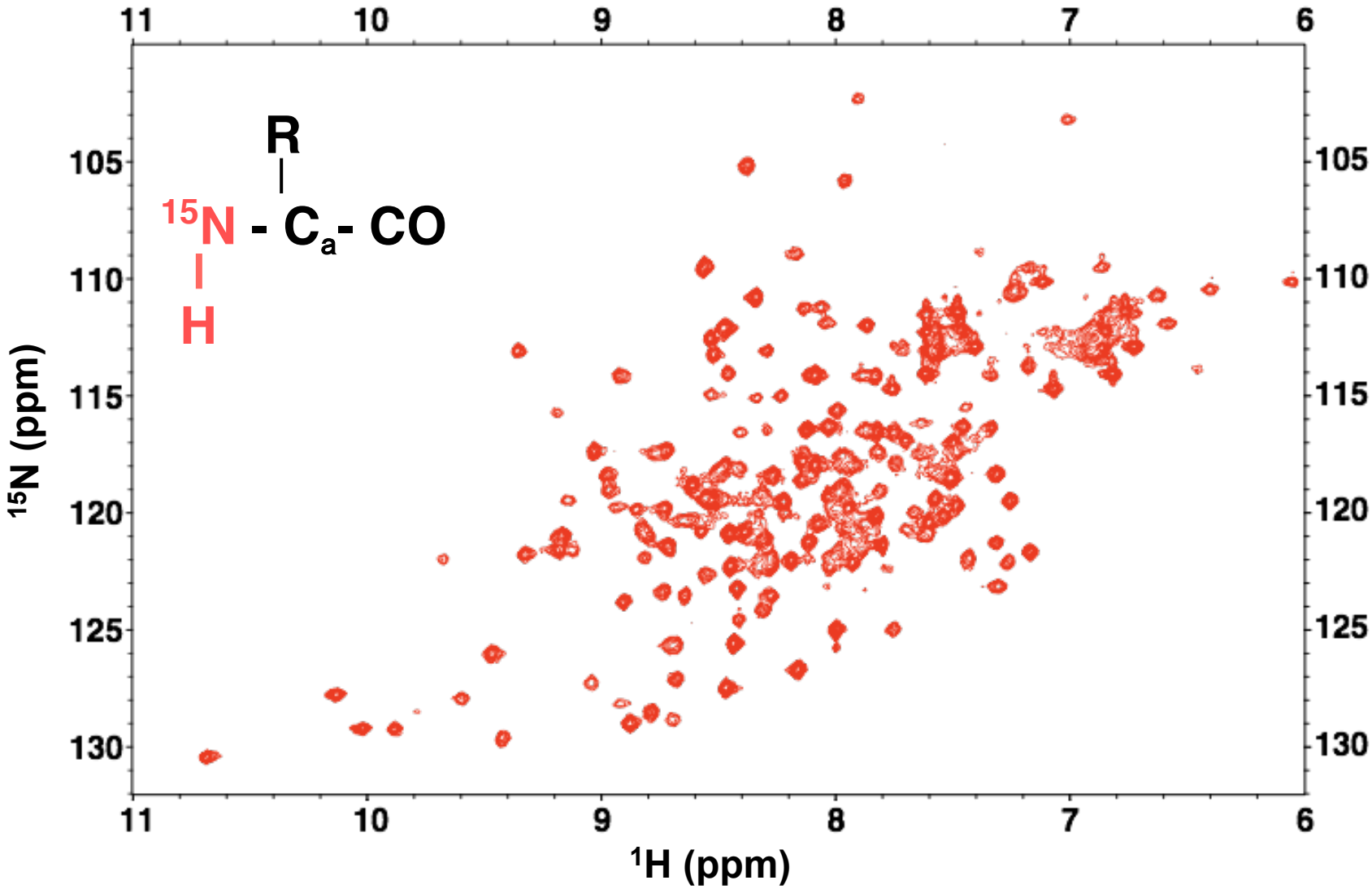
Bacterial expression:
Minimal media, ^{15}N NH_4Cl or
^{13}C glucose as sole nitrogen and
carbon source

Amino acid-type labeling
Auxotrophic or standard strains
(ei, BL21(DE3) depending on scheme

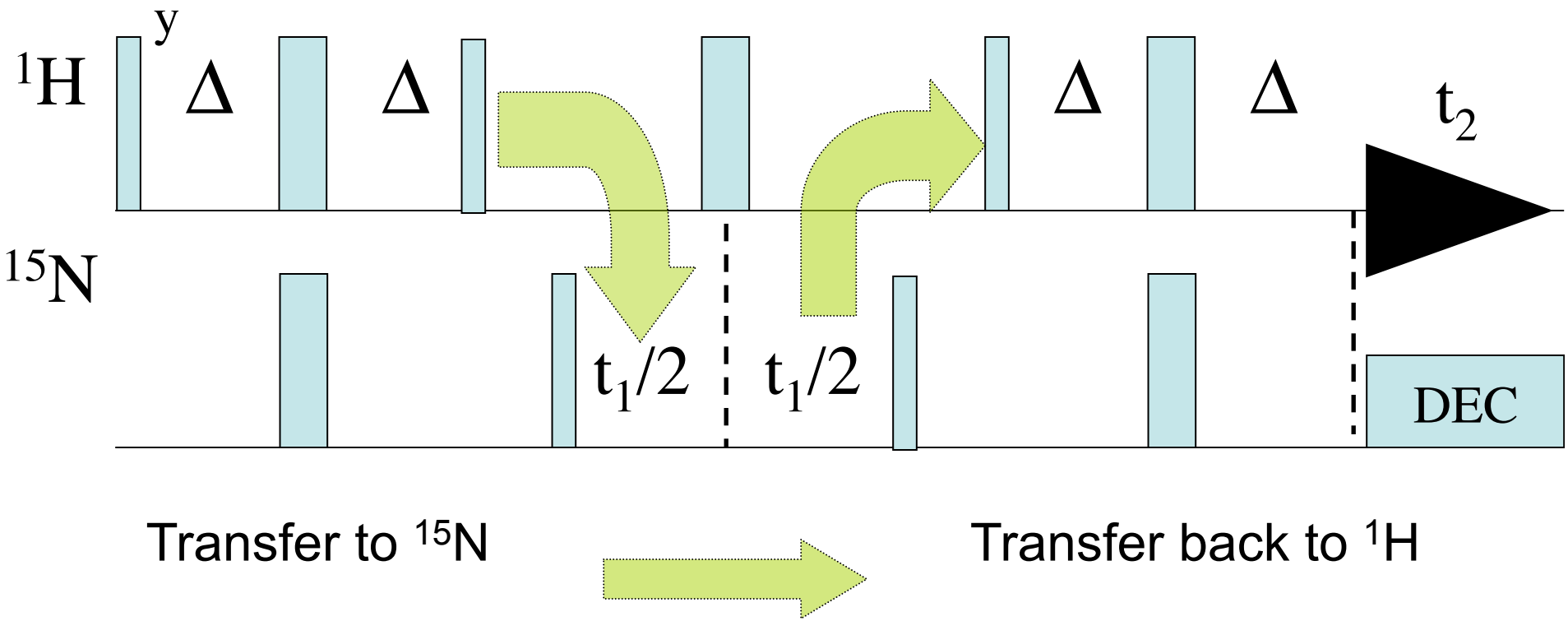
Labeling post purification ; reductive
methylation of lysines

Results in additional spin-1/2 nuclei which can be used as probes

The HSQC is an NH chemical shift correlation map



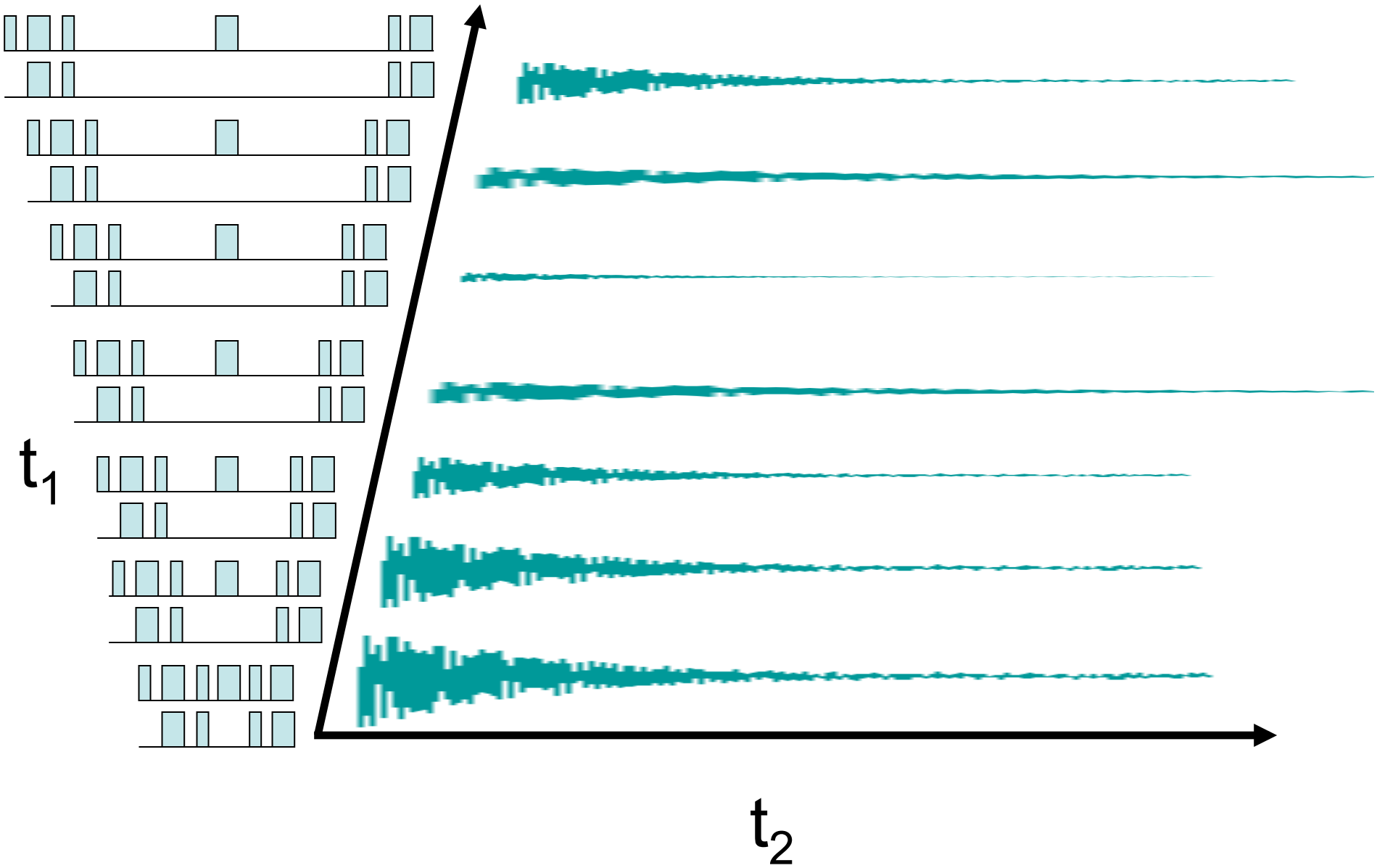
An overview of the HSQC



Encode ^{15}N chemical shift for time t_1

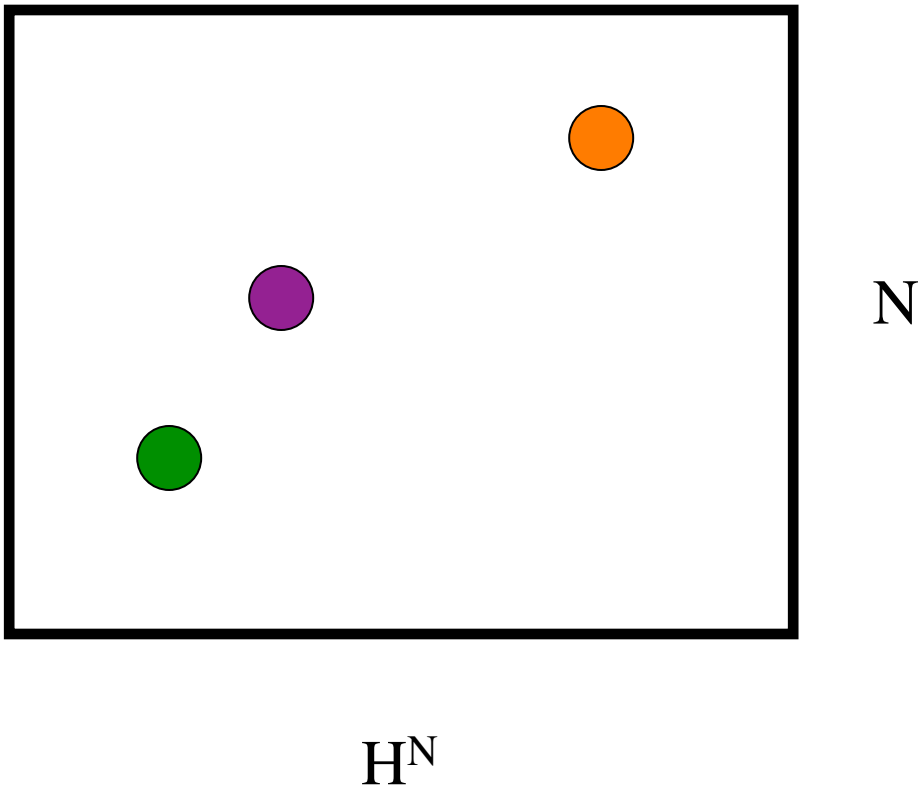
Bodenhausen & Ruben

2D Time-Domain Data

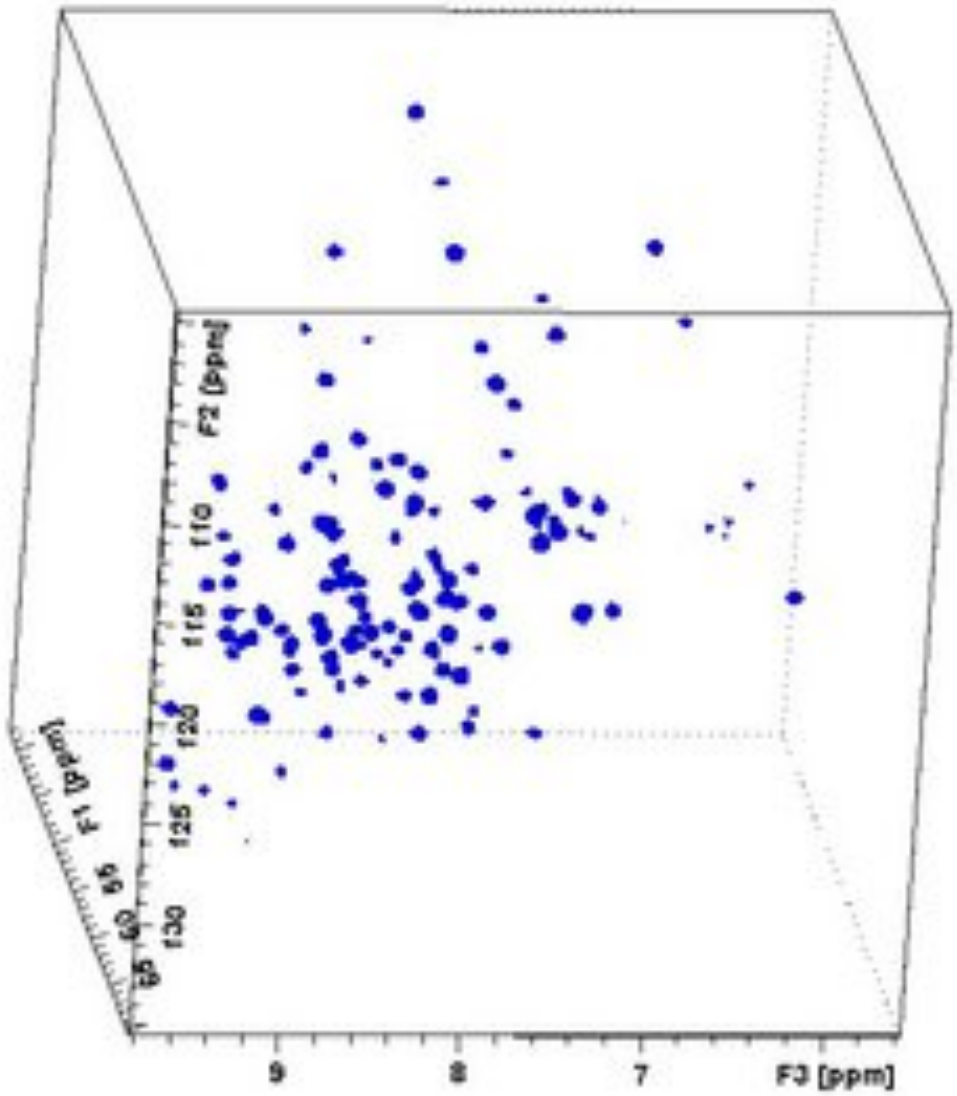


Some data shuffling then 2D FT =the HSQC Spectrum

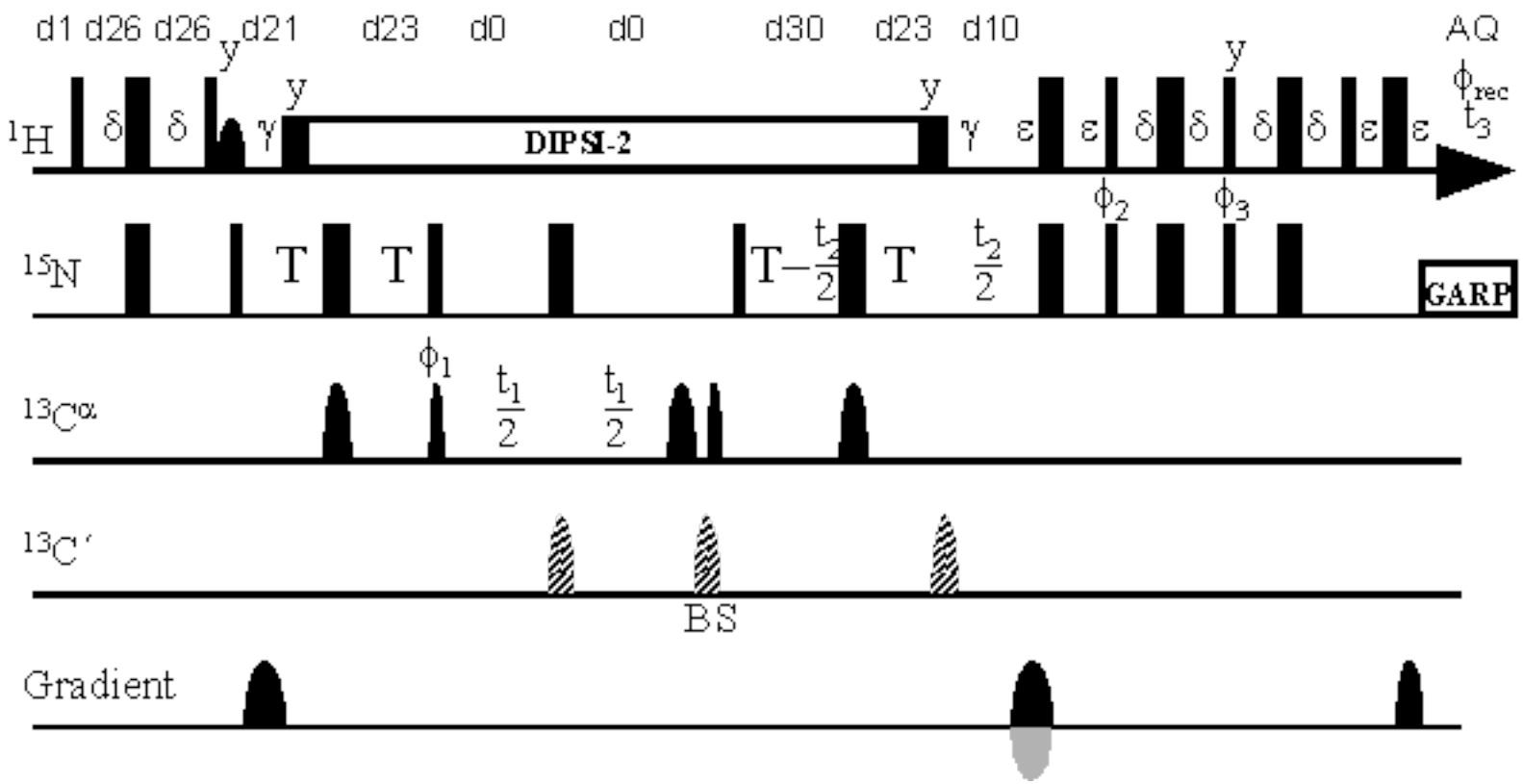
$$\mathrm{Re}\,[S'(\nu_1, \nu_2)] = A_1^N A_2^H$$



3D Dimensional NMR

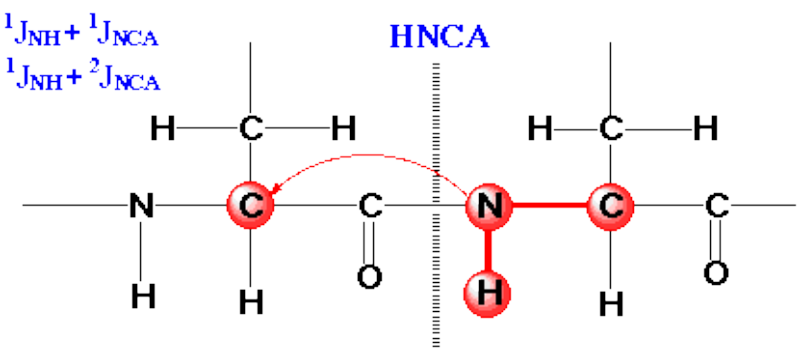


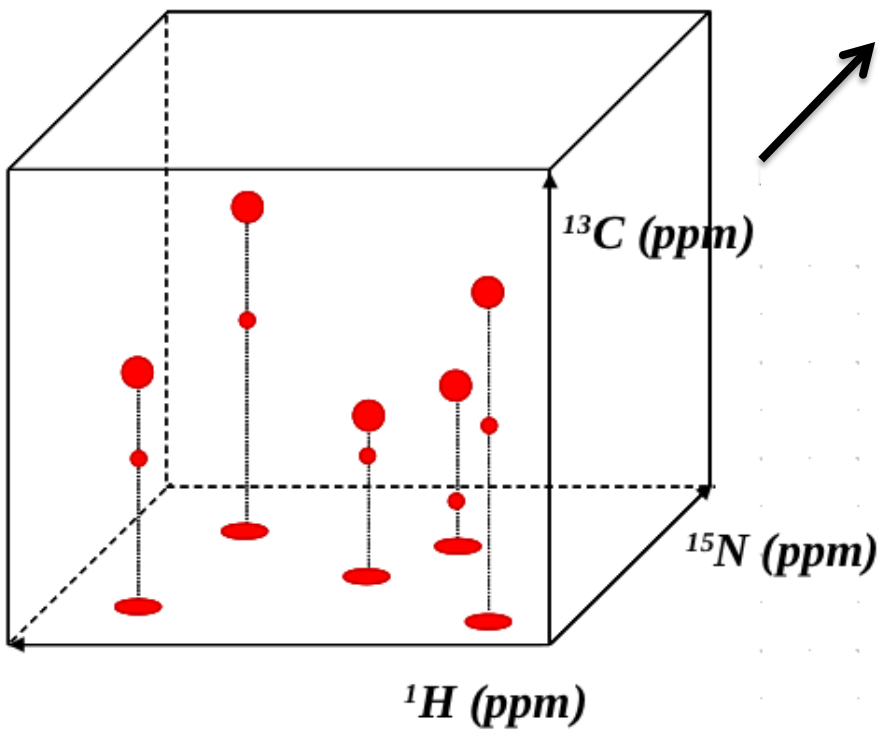
Resonance Assignments from Triple Resonance Experiments

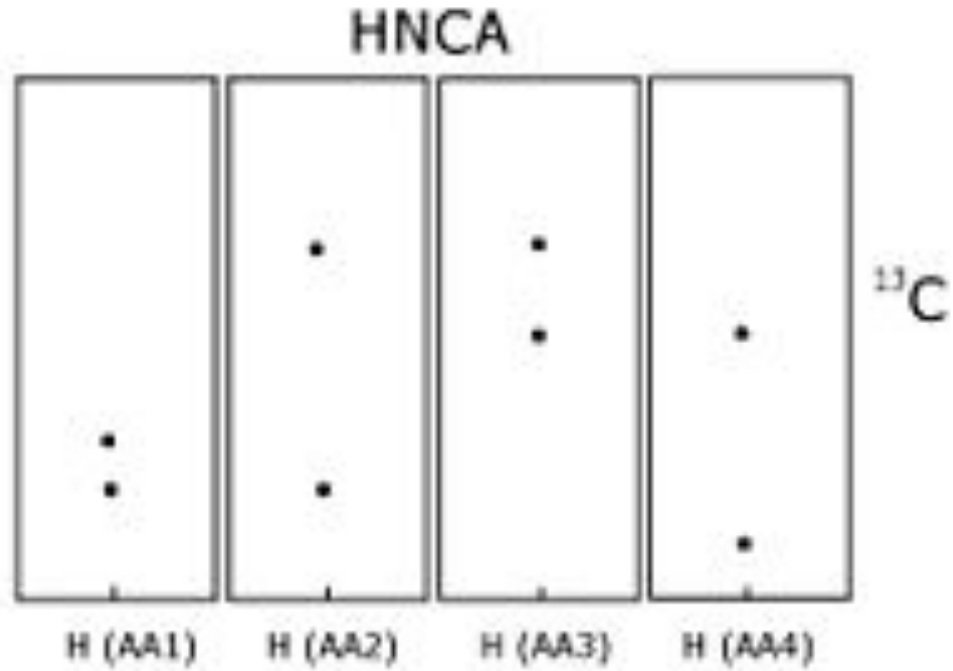


The 3D HNCA Experiment

Backbone Resonance Assignments from HNCA

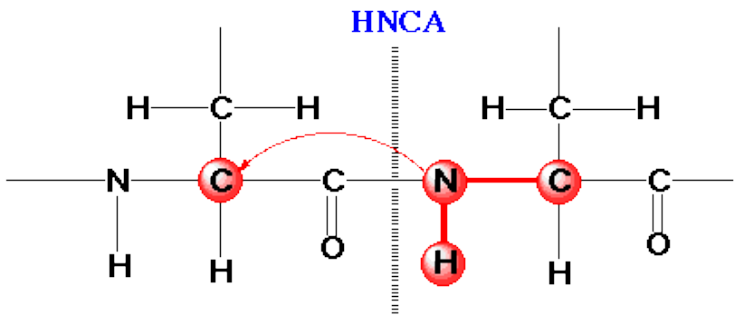


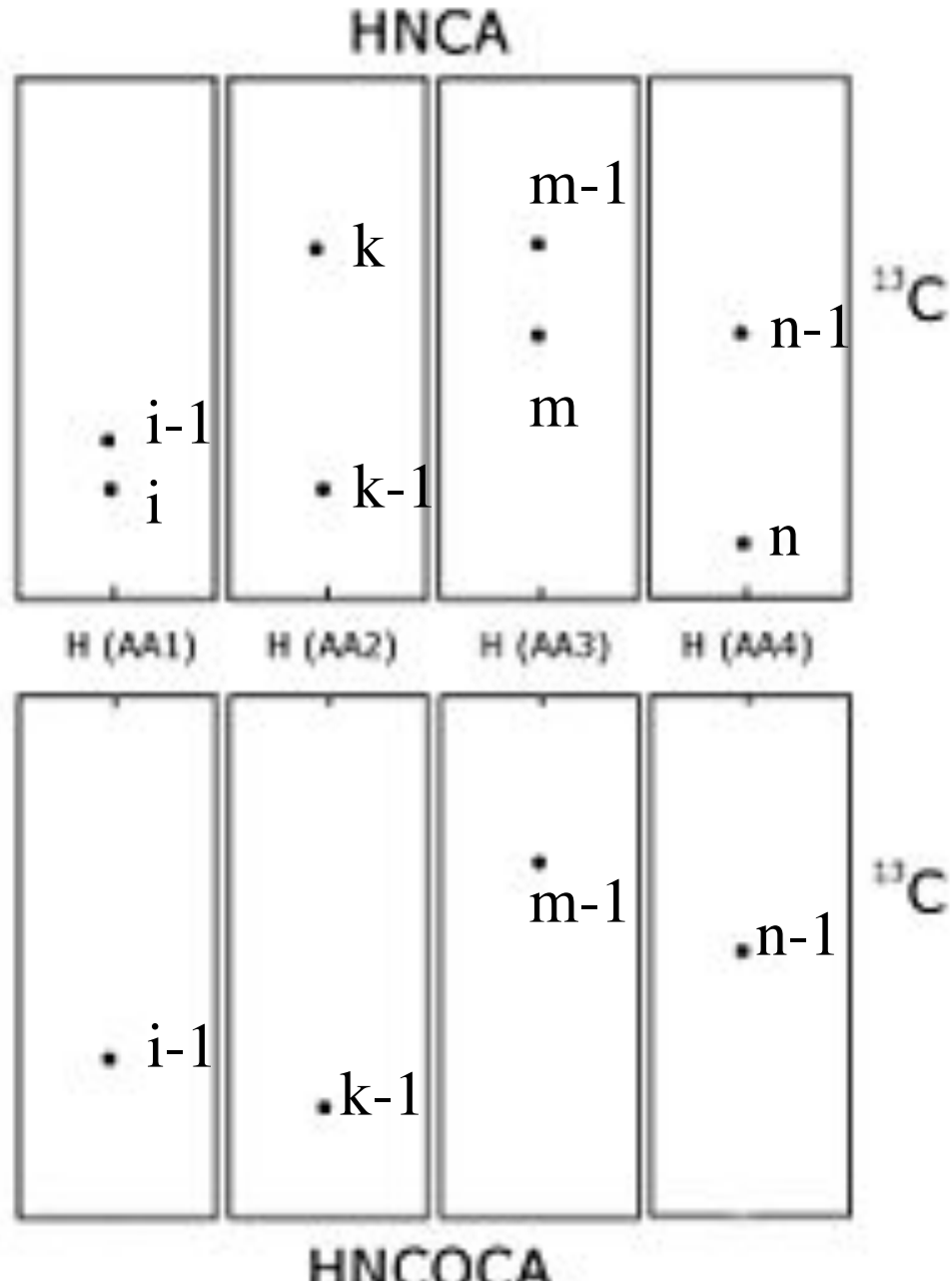




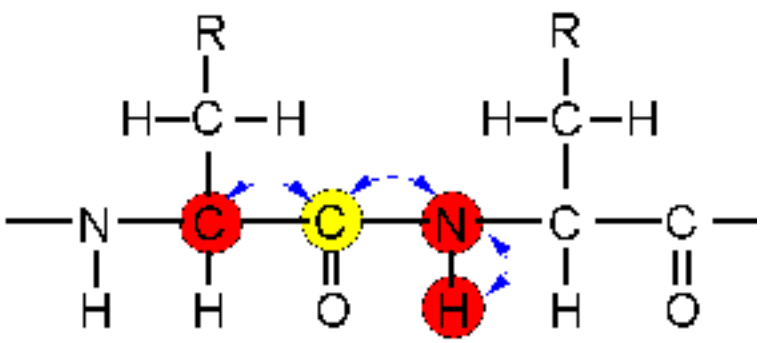
Triple Resonance Pairs

Residue i-1 Residue i





HN(CO)CA



sequential correlation only

THE RANGE OF ^{13}C CHEMICAL SHIFTS OBSERVED FOR EIGHT DIFFERENT PROTEINS

Res.	α	β	γ		δ	ε
Gly	42-48					
Ala	49-56	18-24				
Ser	55-62	61-67				
Thr	58-68	66-73	19-26			
Val	57-67	30-37	16-25			
Leu	51-60	39-48	22-29		21-28	
Ile	55-66	34-47	25-31	14-22	9-16	
Lys	52-61	29-37	21-26		27-34	40-43
Arg	50-60	28-35	25-30		41-45	
Pro	60-67	27-35	24-29		49-53	
Glu	52-62	27-34	32-38			
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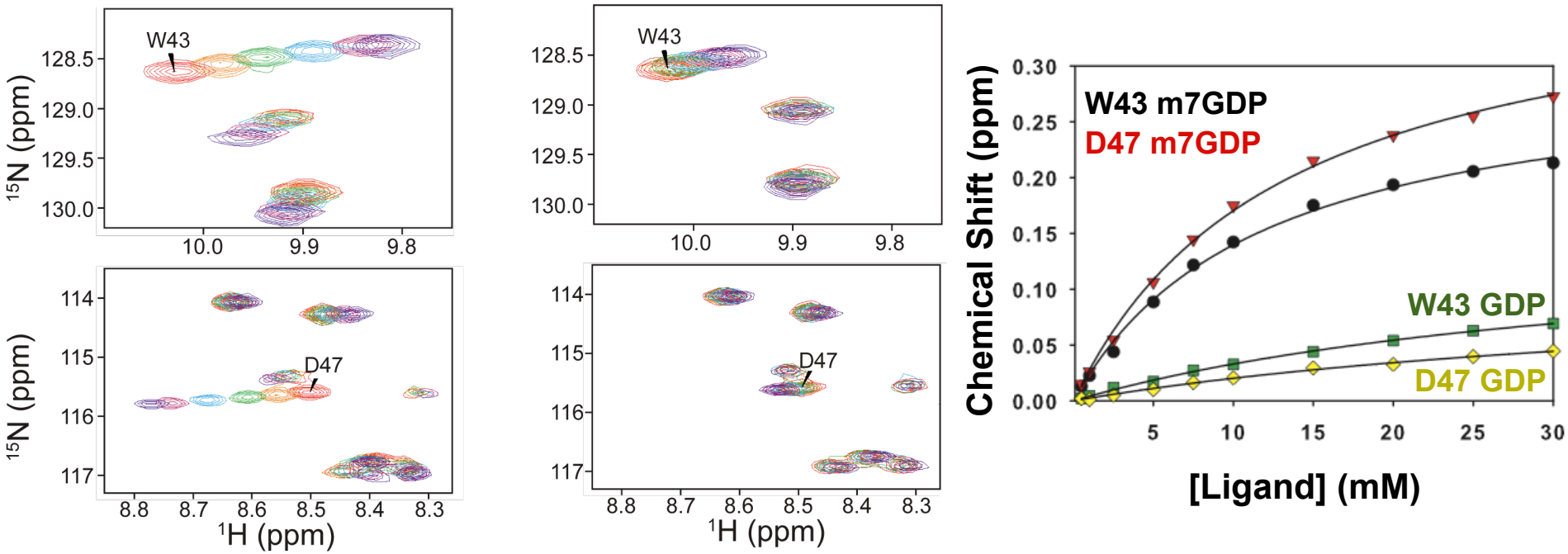
.	.	.	.		.	.

WAGNER AND BRUHWILER, 1986…et al.

Or http://www.bmrb.wisc.edu/ref_info/statsel.htm

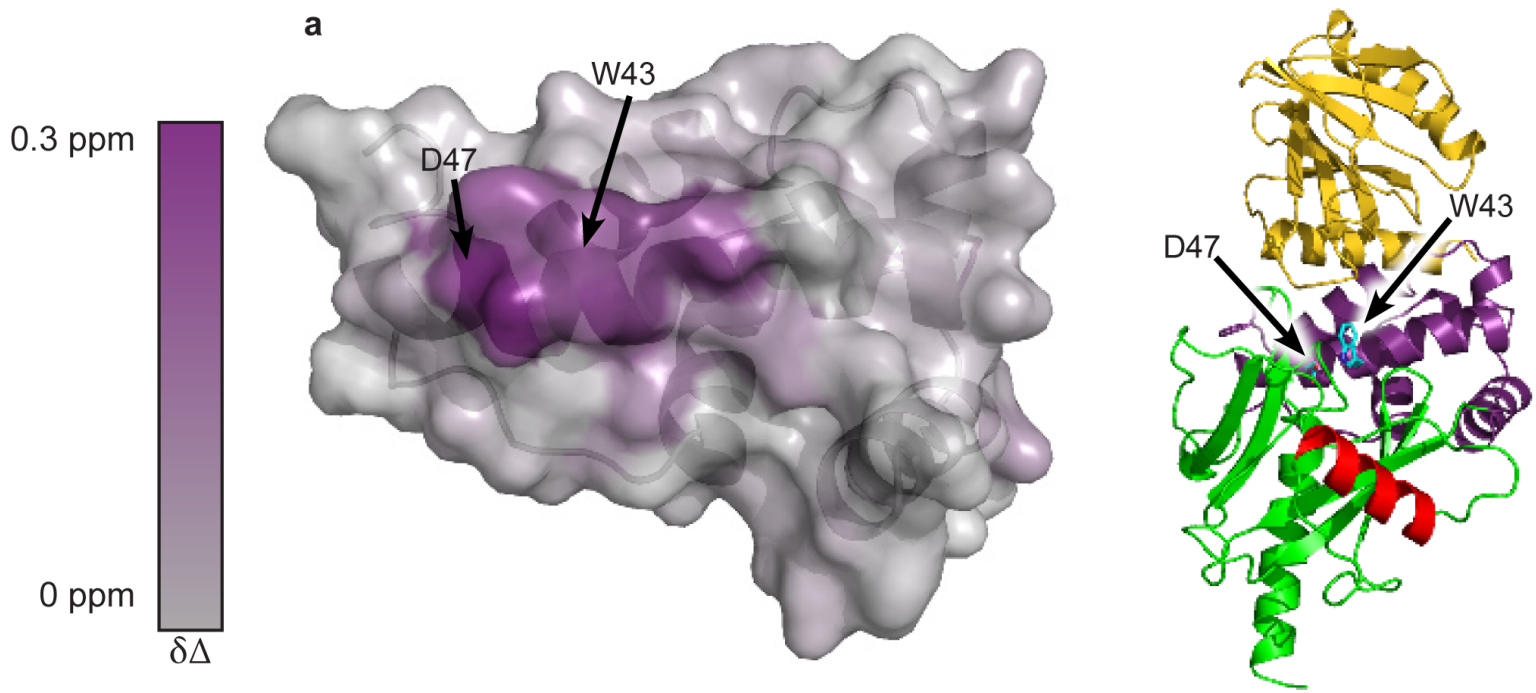
Part II:
Macromolecular Interactions Detected by NMR

Binding of nucleotide to protein



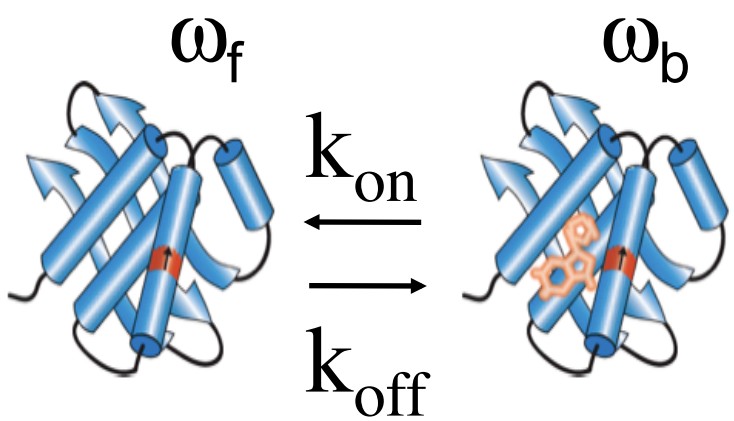
Dose dependent resonance shifts can be fit to obtain Kd

Shifts may be color coded onto surface to identify ligand binding site



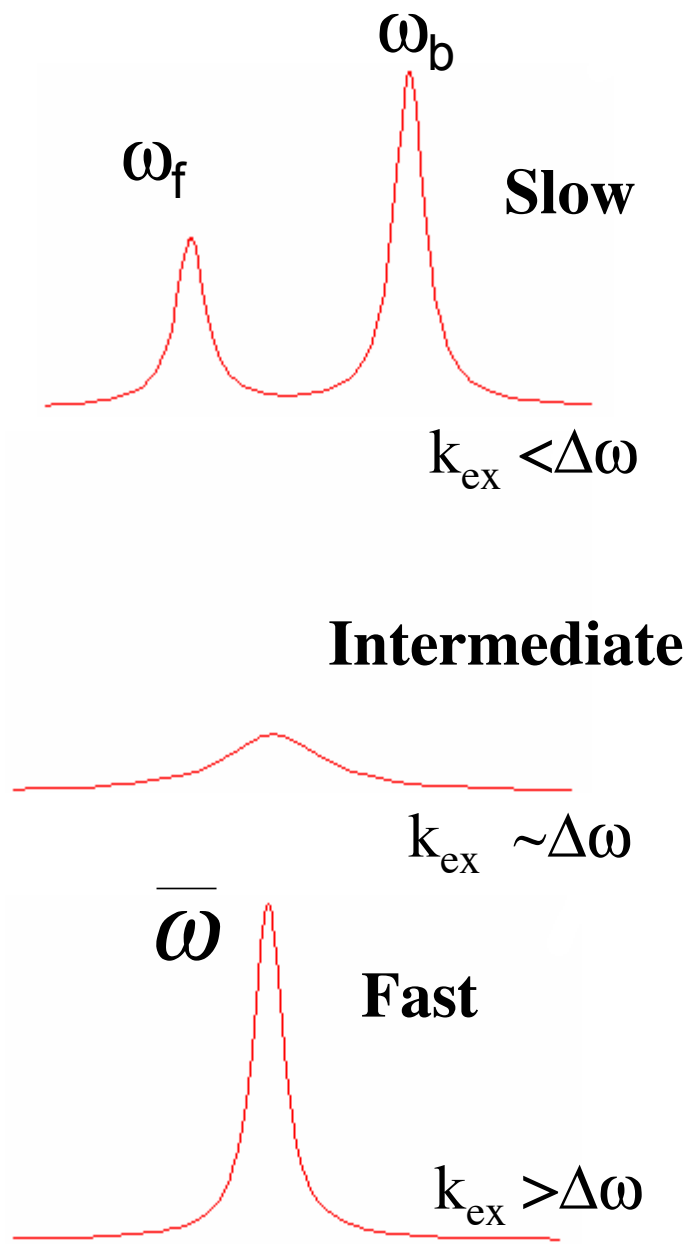
Caveats?

NMR to monitor ligand binding



$$k_{ex} = k_{on}[L] + k_{off}$$

$$\Delta\omega = \omega_f - \omega_b$$

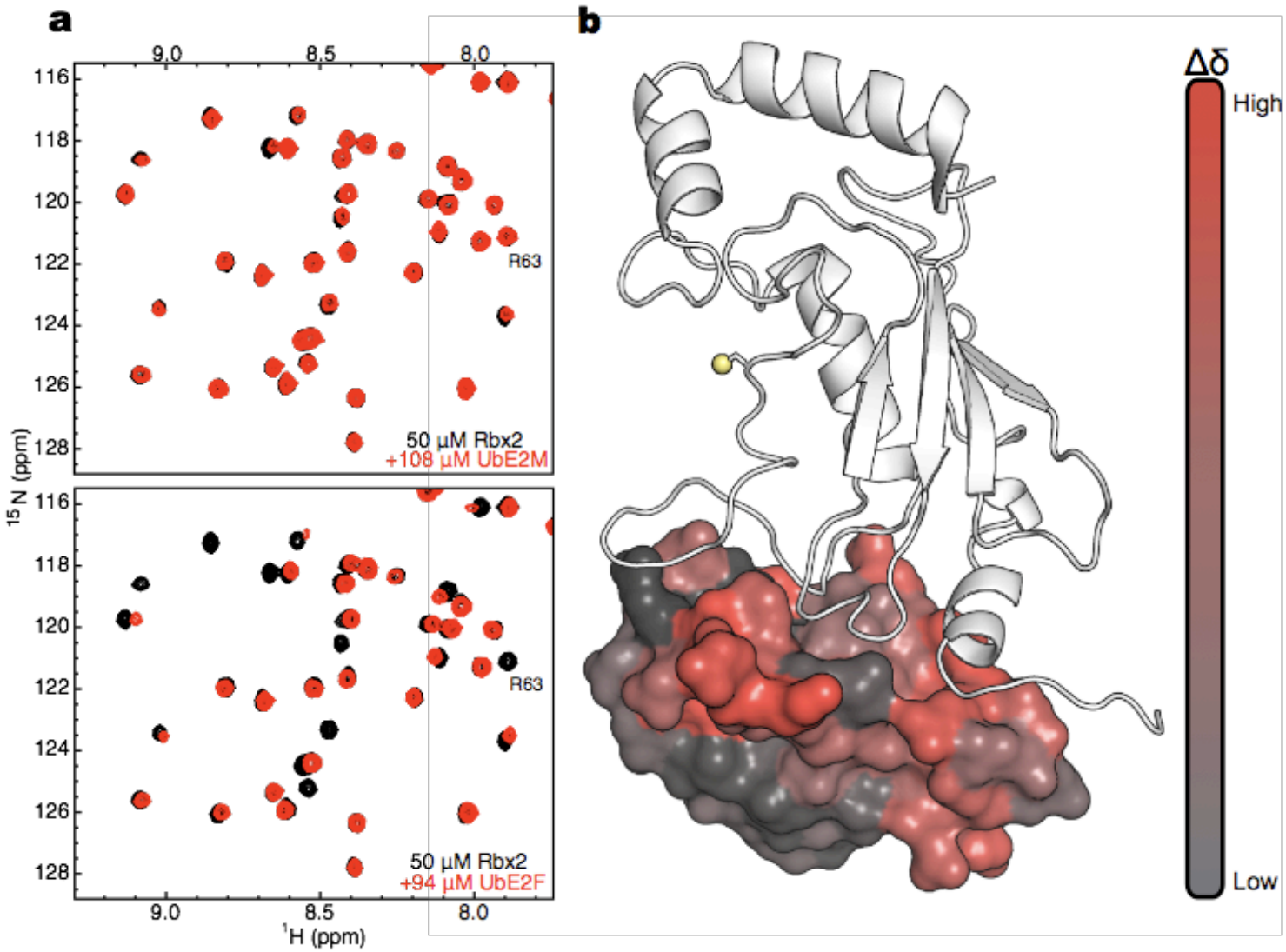


Fraction bound of labeled protein

$$P_b = \frac{\overline{\omega} - \omega_f}{\omega_b - \omega_f} = \frac{[L]}{[L] + K_d}$$

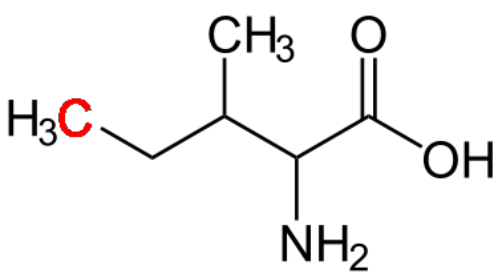
$\overline{\omega}$: observed chemical shift

Monitoring Protein/Protein Interactions by HSQC

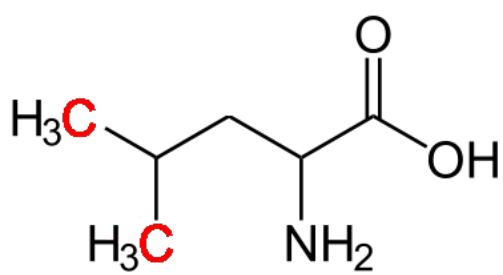


Sparse Labeling to Simplify Spectra

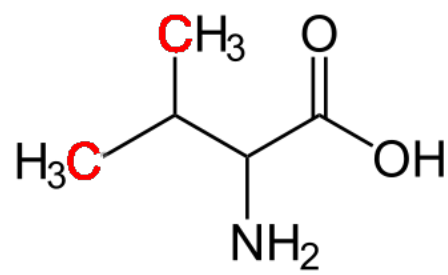
Selectively label R group methyls with C-13 (NMR visible)



Isoleucine



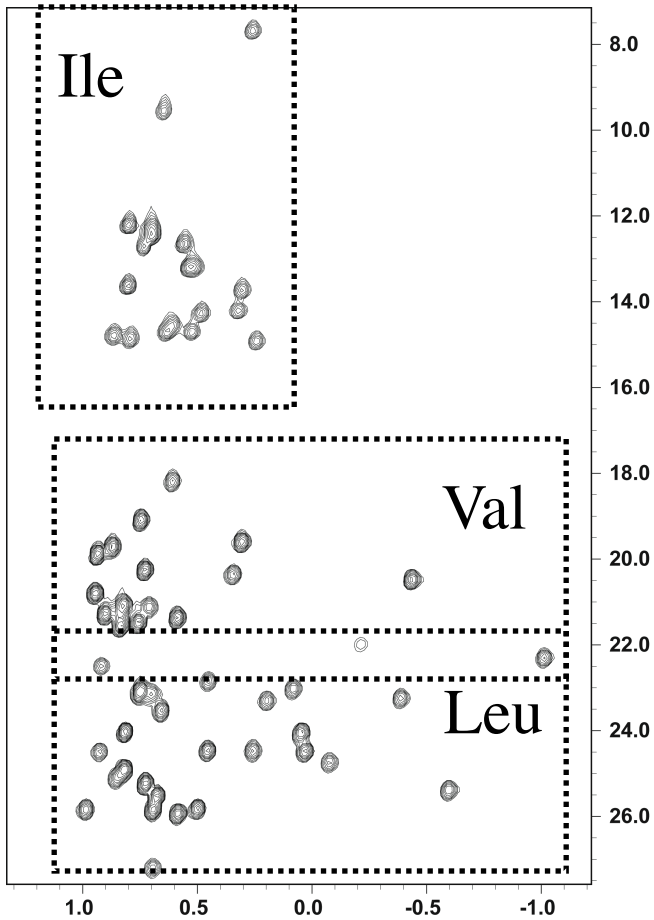
Leucine



Valine

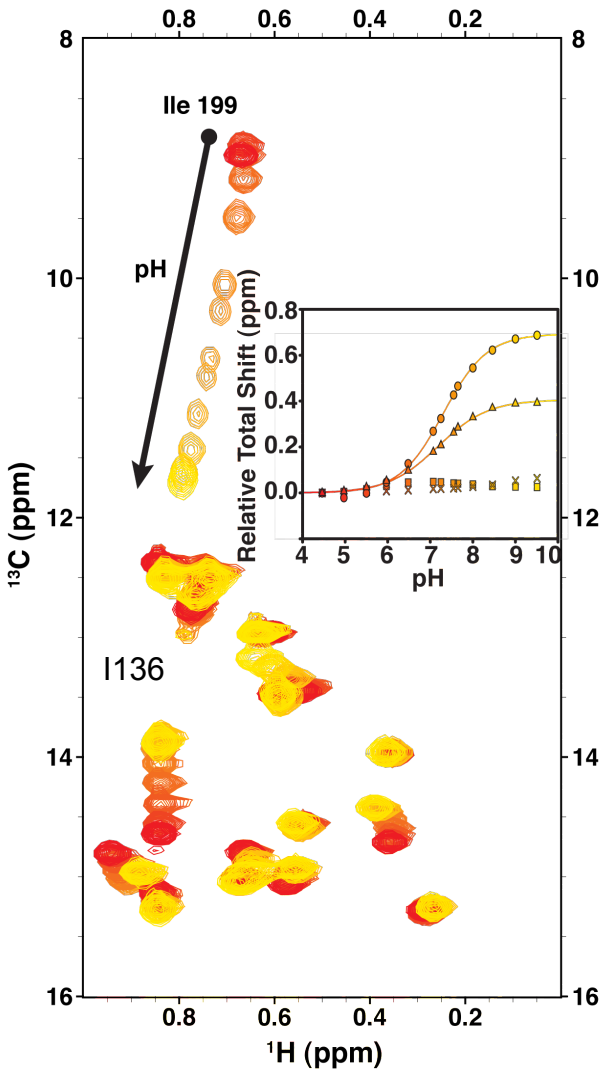
(add alpha-ketoacid precursors to ILV 30 minutes prior to induction)

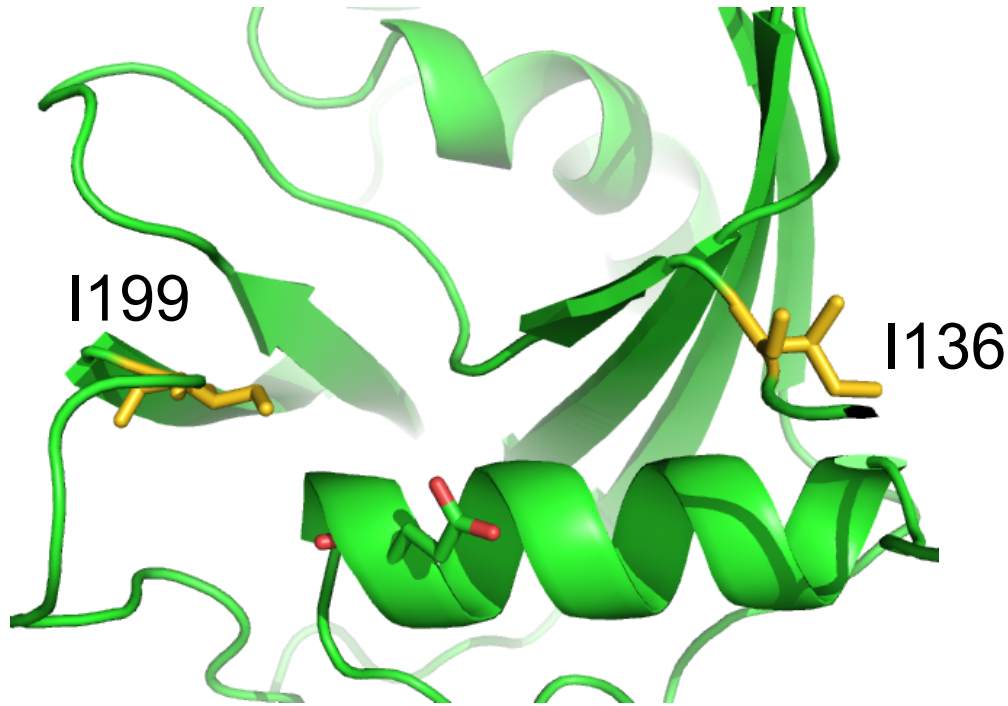
^{13}C HSQC of ILV labeled protein



Measuring pKa by NMR

(pH 4.5 - 9.5)





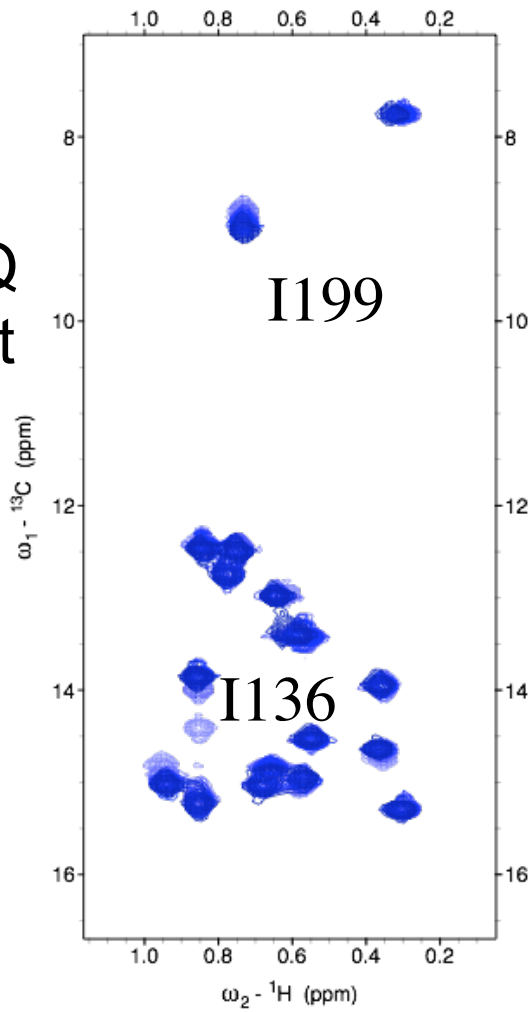
pKa of 7.2, elevated for Glu

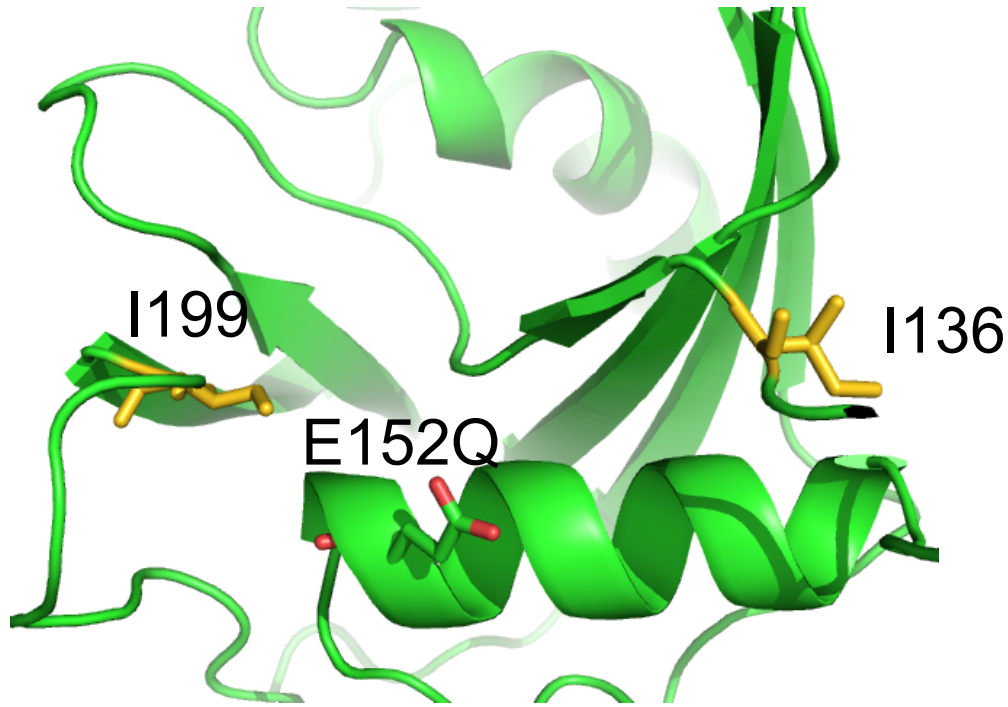
Aglietti et al, Structure 2013

Identification of titratable residue by site-directed mutagenesis and NMR

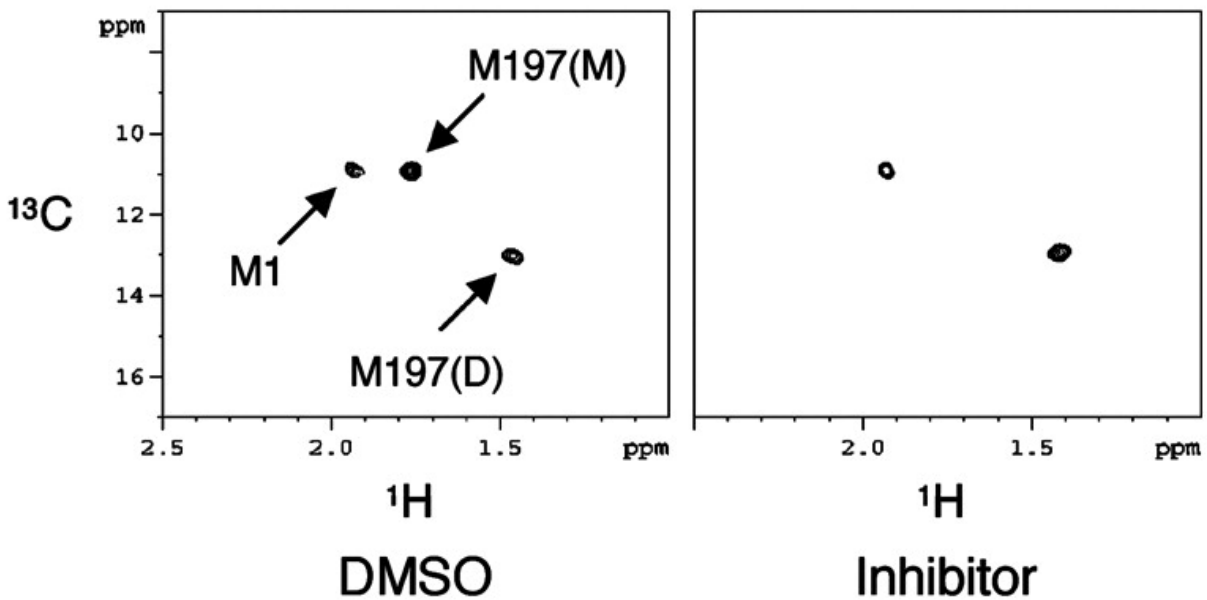
(pH 4.5 - 9.5)

E152Q mutant





Example of slow exchange: monomer-dimer equilibrium



Inhibition of KSHV Pr stabilizes the dimeric conformation

Methionine specific labeling simplifies analysis

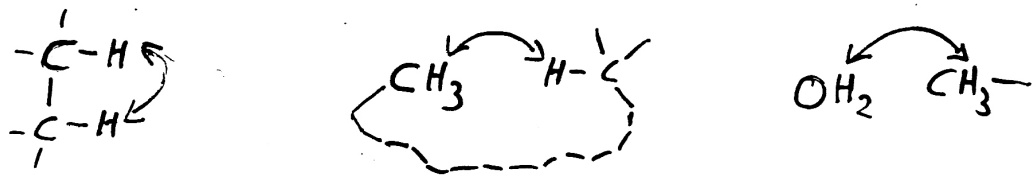
Marnett A. B. et.al. PNAS 2004;101:6870-6875

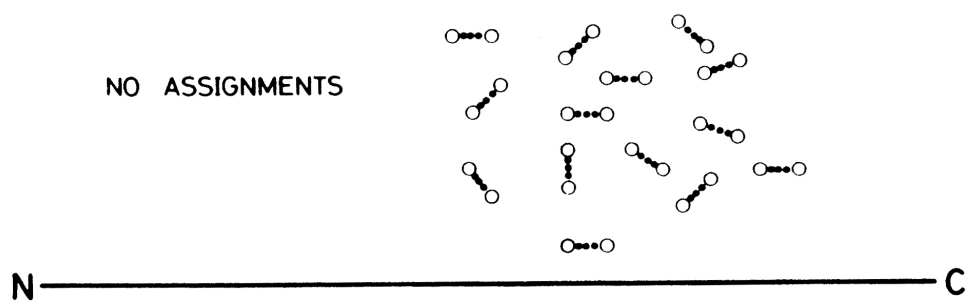
Part III: Structure by NMR

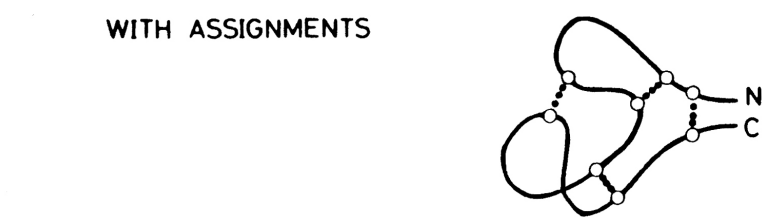
Calculating 3D structures: we need distance measurements & assignments

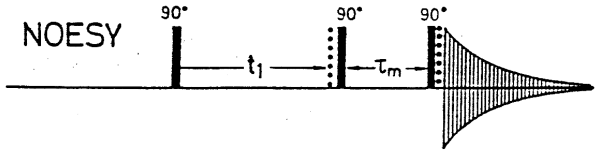
$$NOE = f(\tau_c) \cdot \frac{1}{r^6}$$

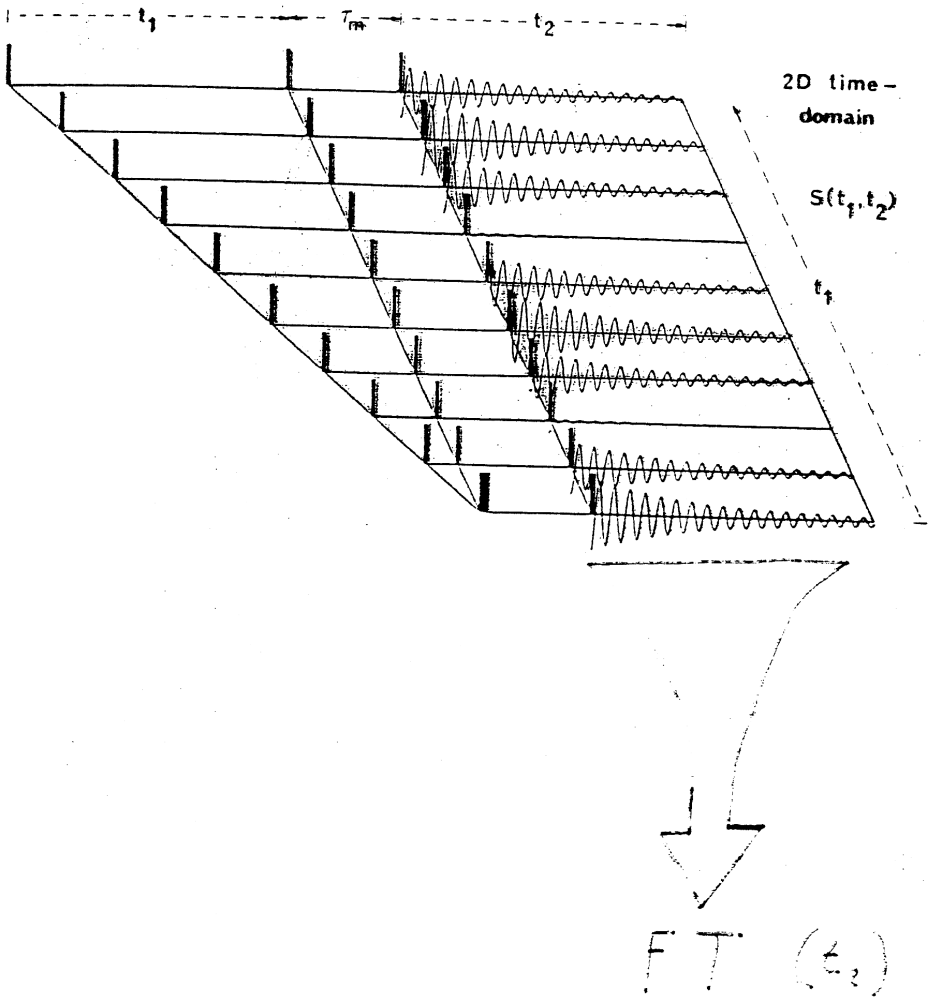
We can see distances that are $\leq 5\,\mathring{A}$



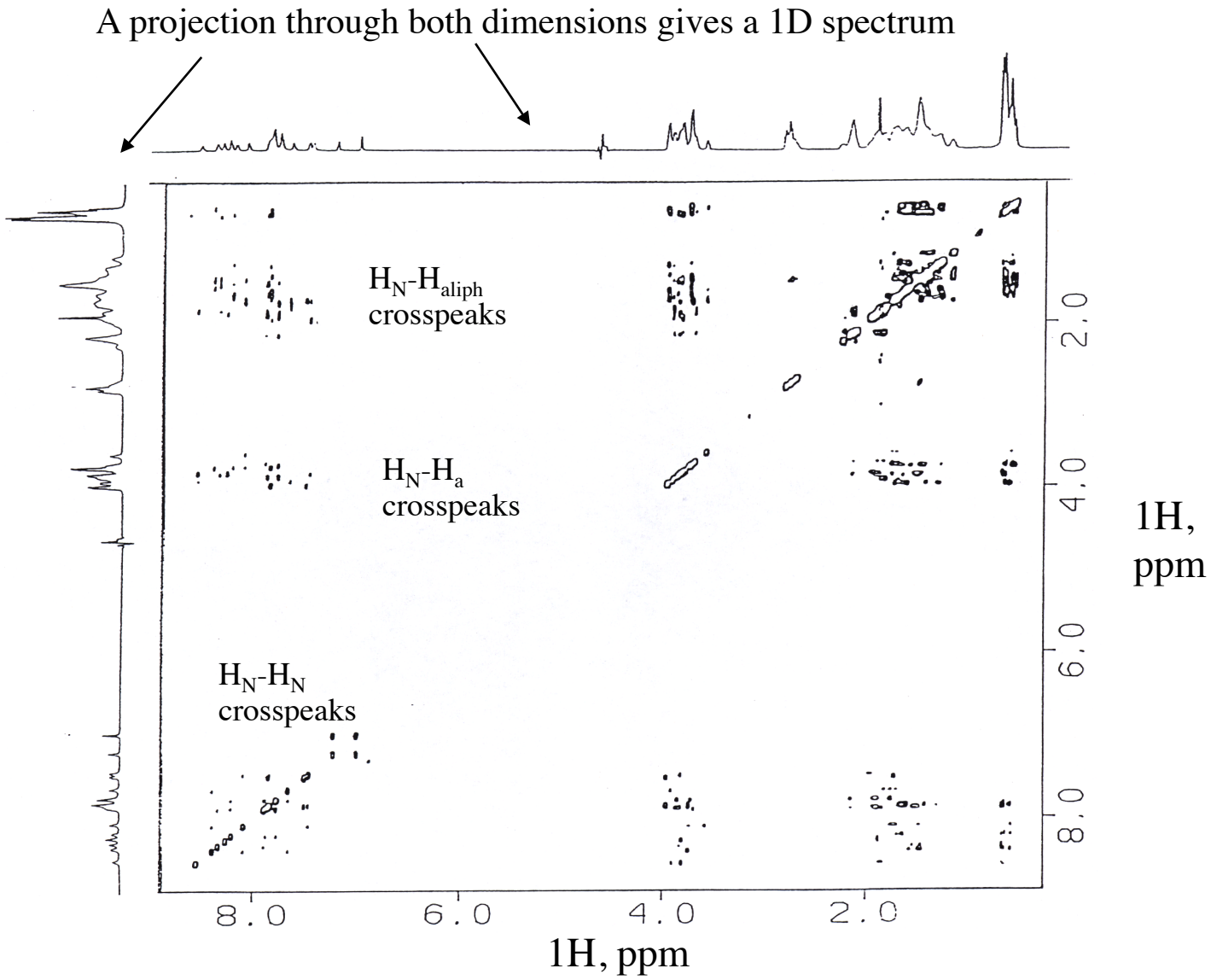





NOESY
90°
90°
90°
t_1
τ_m


t_1
τ_m
t_2
2D time-domain
$S(t_1,t_2)$
t_1
FT (t_2)

A Real 2D NOE Experiment of a Small Peptide

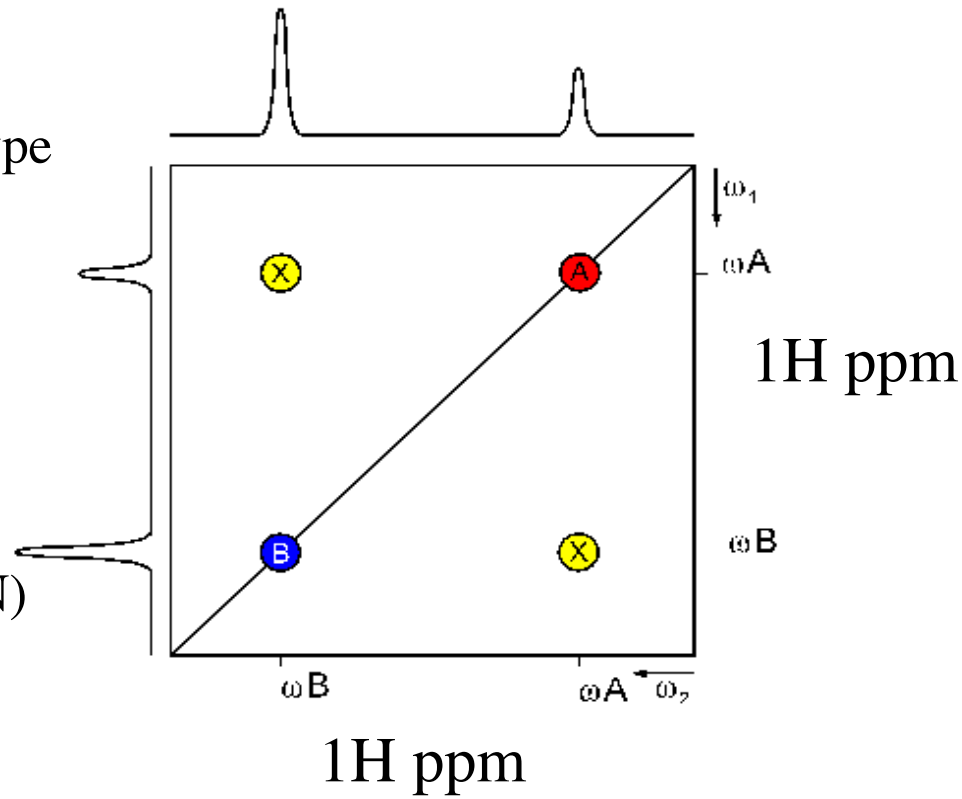


Interpretation of 2D NMR Spectra

- Crosspeaks are a measure of some type of interaction between 2 spins (NOE, J-coupling....)

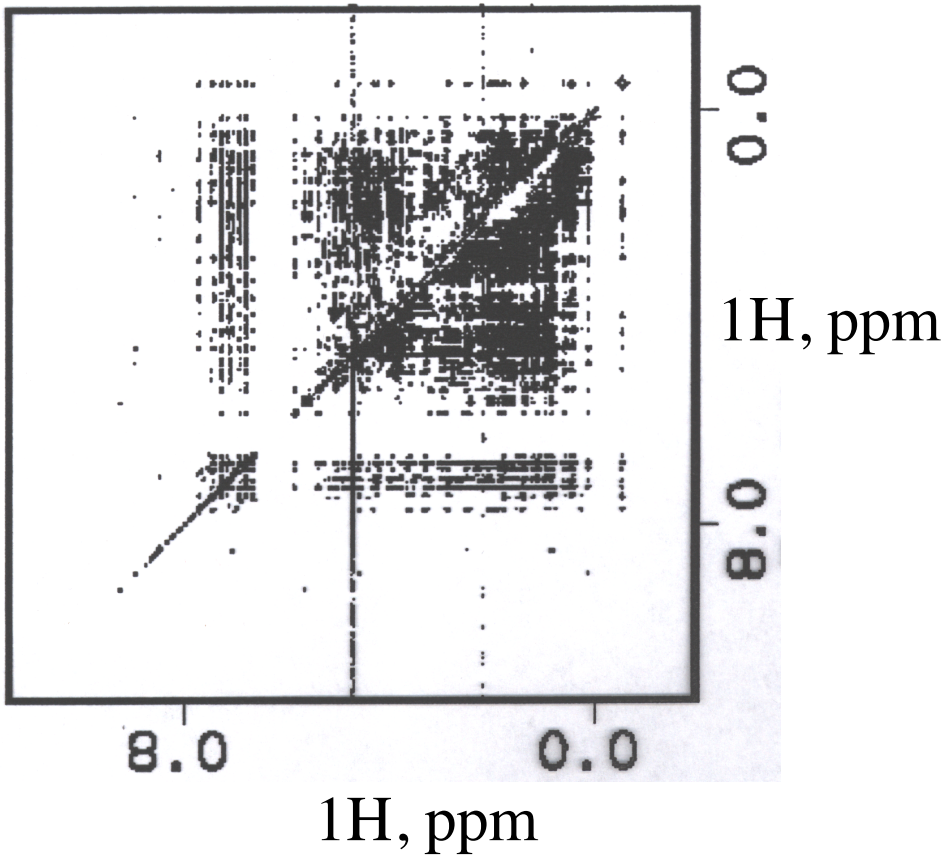
- The intensity of the crosspeak often quantifies the interaction.

- A heteronuclear experiment (1H-15N) would not have diagonal crosspeaks.



Higher Dimensionality 3 and 4D Heteronuclear Experiments on Isotopically Labeled (15N-13C) Proteins

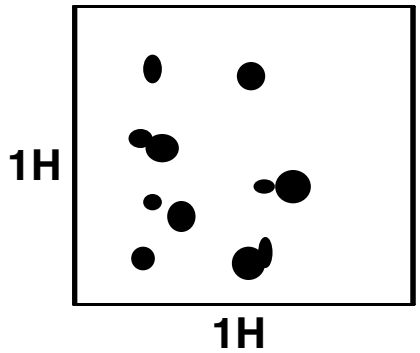
2D NOESY of a 76 residue protein homodimer (effectively 18kD) in D_2O



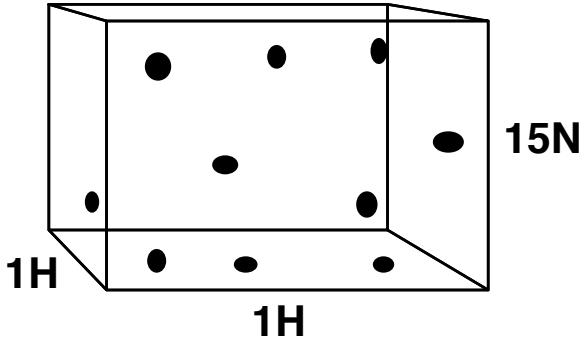
In practice, even small proteins have very crowded 2D spectra making assignment very difficult. In this case the fact that it is in D2O simplifies the spectra because the amide protons exchange for deuterium and are not visible.

Benefit of C13 and N15 labeling of Proteins for NMR

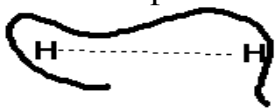
Higher Dimensionality (3 and 4D) Experiments Reduce Overlap Compared to 2D Experiments

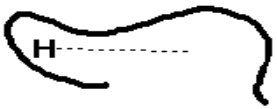


2D nOe Expt. on unlabeled protein



3D nOe Expt. on N15-labeled protein



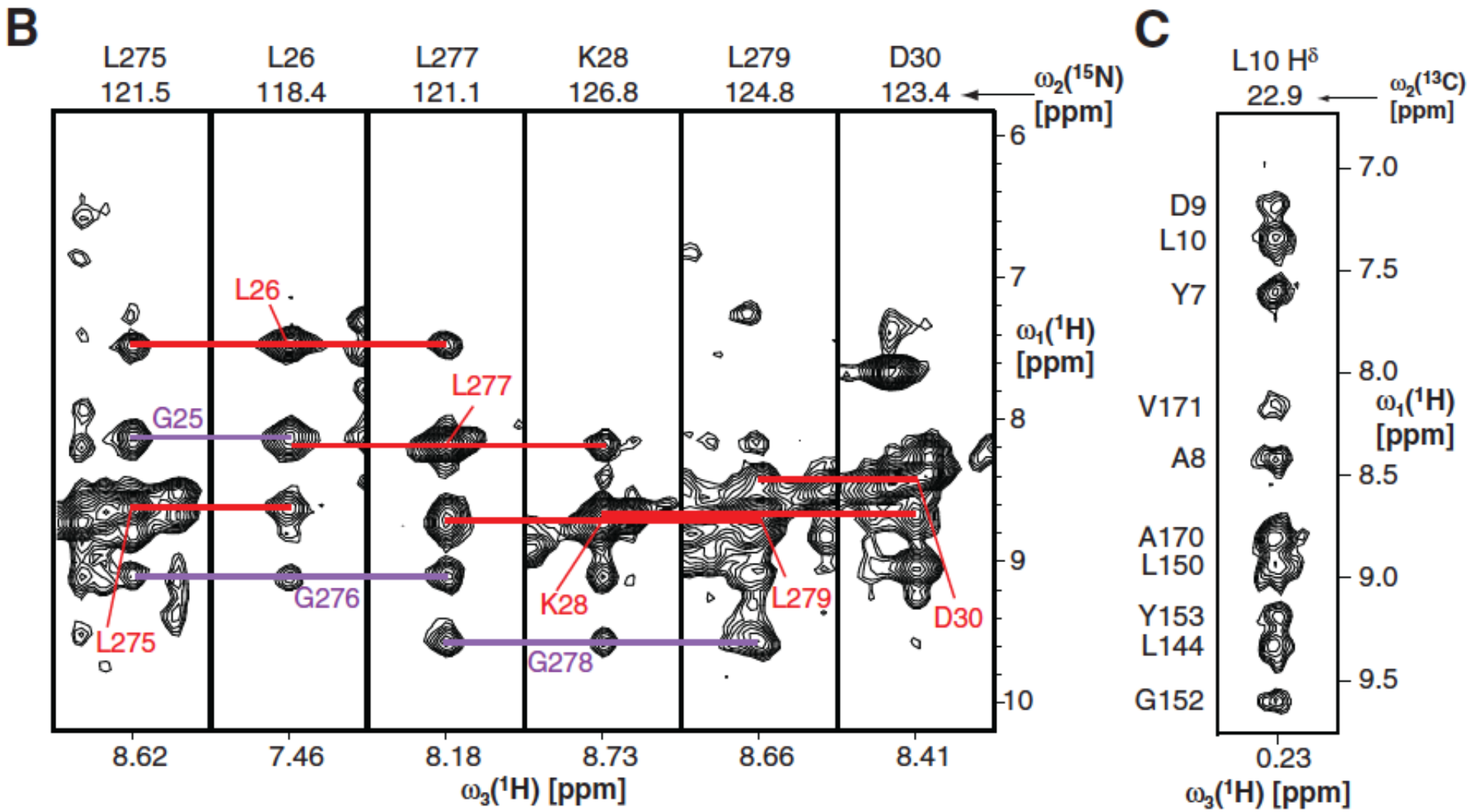


Many More Types of Experiments Can be Done on Isotopically Labeled Protein

nOes between Protons Attached to N15 and Protons Attached to 13C

nOes between Protons Attached to 13C and Protons and Attached to 13C

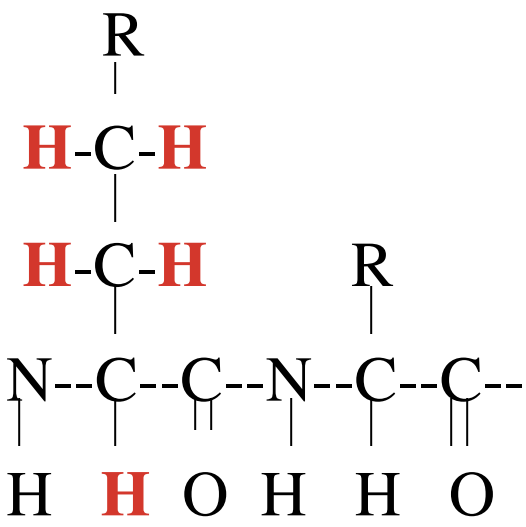
Examples of 15N and 13C dispersed NOESY



15N NOESY-HSQC

13C NOESY-HSQC

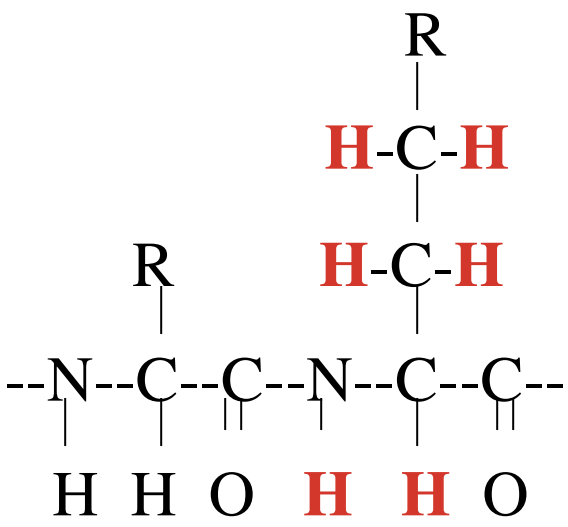
Side-chain protein assignments



H(CCO)NH-TOCSY

i - 1 res.

All Carbon's H's at i-1 to N-H pair.

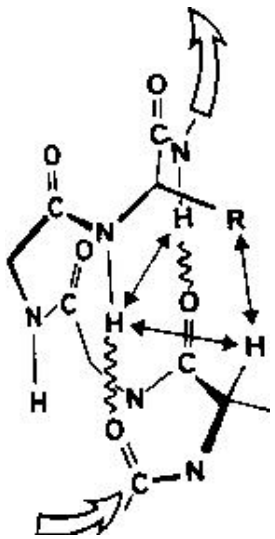


15N-TOCSY i res.

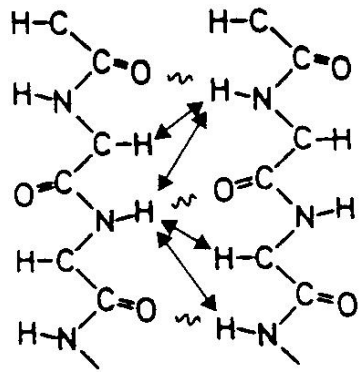
All H's at i to N-H pair.

TOCSY methods relies on through-bond J Couplings

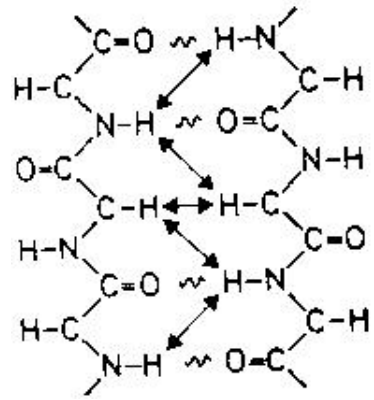
Close interatomic distances in secondary structures



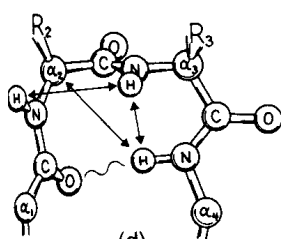
alpha-helix



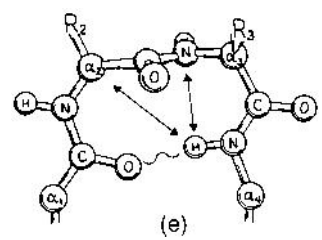
parallel beta-sheet



antiparallel beta-sheet



type I turn

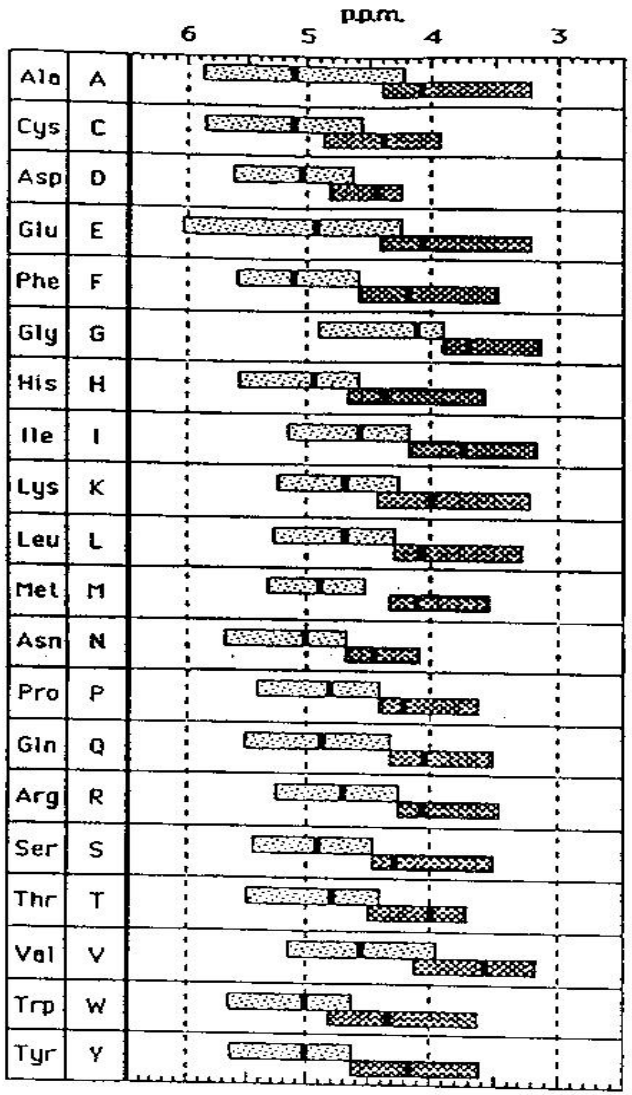


type II turn

H^a chemical shifts and secondary structure

- **the figure at right shows distributions of H^a chemical shifts observed in sheets (lighter bars) and helices (darker bars).**

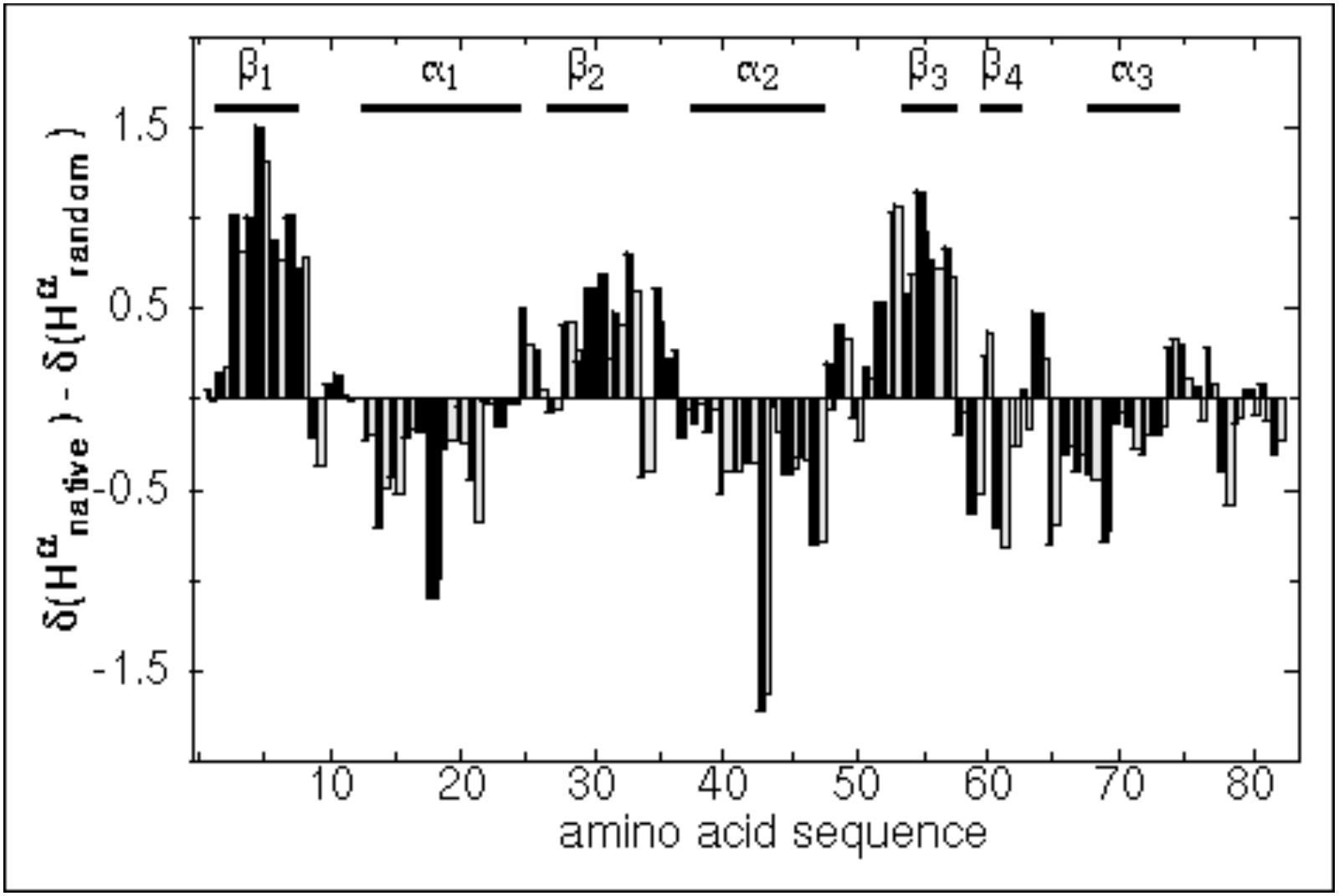
- **H^a chemical shifts in a–helices are on average 0.39 ppm below "random coil" values, while b-sheet values are 0.37 ppm above random coil values.**



Wishart, Sykes & Richards

J Mol Biol (1991) **222**, 311 .

Secondary Shift vs Sequence



Reveals secondary structure !

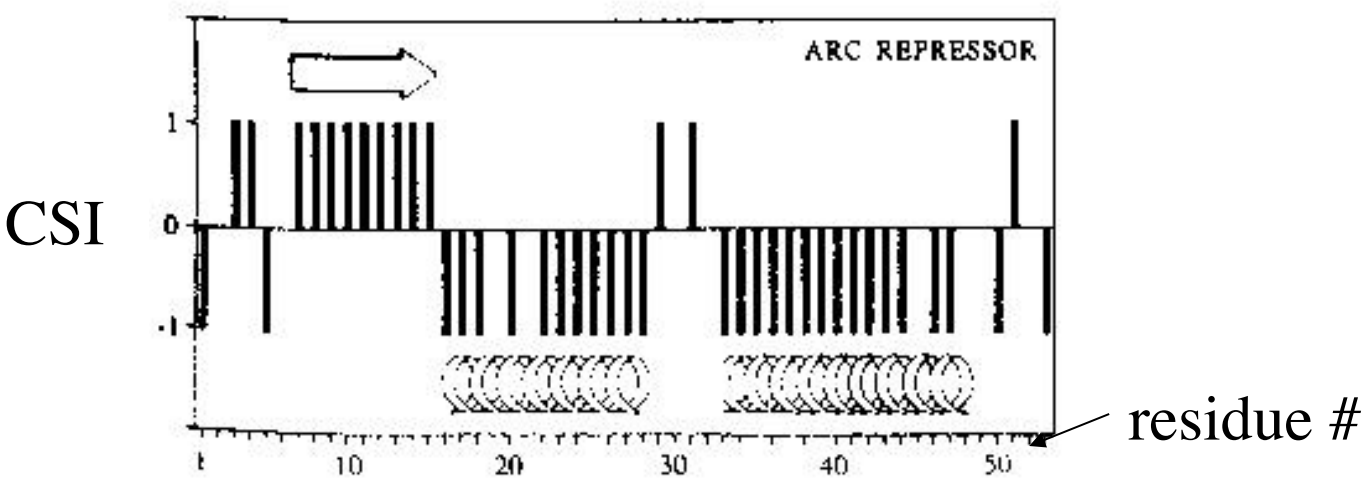
Chemical shift index (CSI)

- trends like these led to the development of the concept of the *chemical shift index** as a tool for assigning secondary structure using chemical shift values.
- one starts with a table of reference values for each amino-acid type, which is essentially a table of "random coil" H^a values
- CSI's are then assigned as follows:

exp'tl H^a shift rel. to reference	assigned CSI
within ± 0.1 ppm	0
>0.1 ppm lower	-1
>0.1 ppm higher	+1

*Wishart, Sykes & Richards *Biochemistry* (1992) **31**, 1647-51.

Chemical shift indices

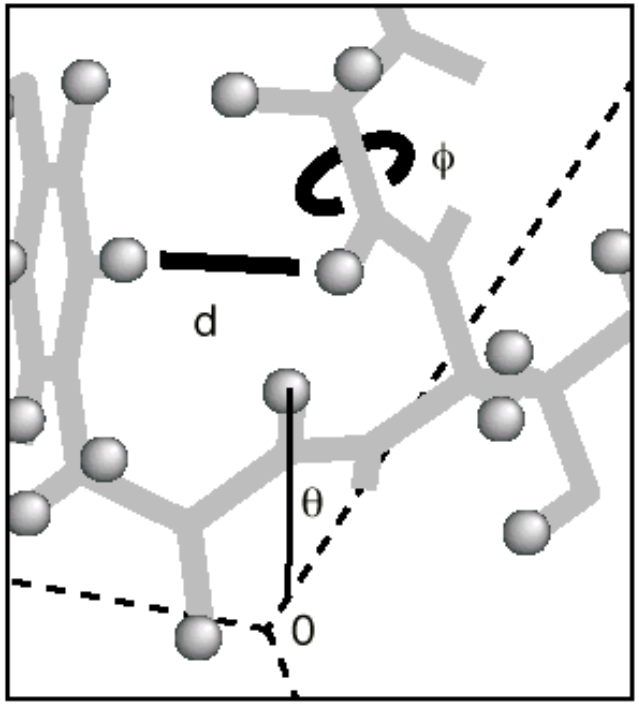


- any "dense" grouping of four or more "-1's", uninterrupted by "1's" is assigned as a helix, while any "dense" grouping of three or more "1's", uninterrupted by "-1's", is assigned as a sheet.
- a "dense" grouping means at least 70% nonzero CSI's.
- other one regions are assigned as "coil"
- **this simple technique assigns 2ndary structure w/90-95% accuracy**
- similar useful relationships exist for $^{13}C^{a}$, $^{13}C^{C=O}$ shifts

3D structure calculation

- NMR provides information about structure
    - chemical shifts <=> local electronic environment
    - coupling constants <=> torsion angles
    - NOE, ROE <=> interproton distances
    - residual dipolar couplings <=> bond orientation
- and dynamics
    - relaxation times
    - NOE, ROE
- Most of the data describe
    - local environment of the protons
    - relative to each other
    - not the global conformation of the molecule

Experimental data from NMR



• Distance

NOE: The distance between i and j is a function of the NOE intensity $\mathbf{D_{ij} \sim C(NOE_{ij})^{-6}}$

H-bonds: Identified by slowly exchanging amide H_N protons

•Angles

Side Chain ? and backbone torsion identified from J-coupling experiments

Chemical Shift also gives Angular Information

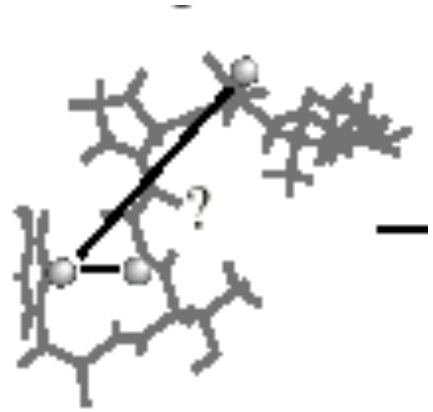
• Residual Dipolar Couplings

Bond Orientations Relative to an Alignment Tensor

3D structure calculation

- Molecular Dynamics with Simulated Annealing starting from random coordinates

- Goal is to minimize the hybrid energy function



Additional
Unambiguous
Experimental
Restraints

E-ForceField

E-NOEs
E-Angles
E-H_bonds
E-Chemical_shift
E-Dipolar_couplings

The hybrid energy function

- Structure calculation = minimization of hybrid energy function (target function) which combines

  1. different experimental data
  2. *a priori* information (force field)

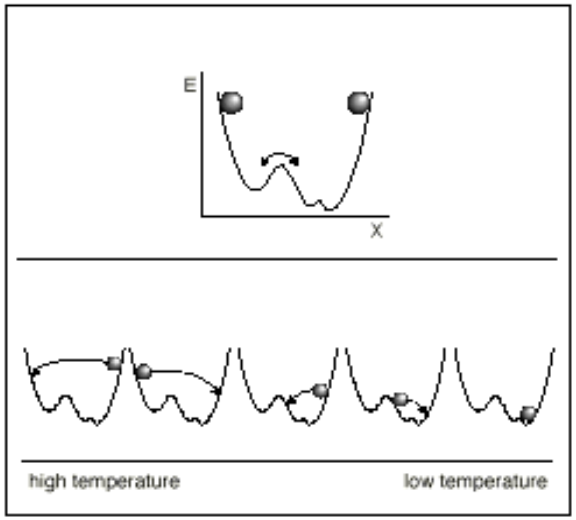
$$
\begin{aligned}
E_{hybrid} &= \textstyle\sum_l w_l E_l = \\
& \quad w_{bond} E_{bond} \\
&+ w_{angle} E_{angle} \\
&+ w_{improper} E_{improper} \\
&+ w_{nonbonded} E_{nonbonded} \\
&+ w_{unambig} E_{unambig} + w_{ambig} E_{ambig} + \ldots \\
&+ w_{torsion} E_{torsion} \\
&+ w_{Jcoup} E_{Jcoup} \\
&+ w_{RDC} E_{RDC} + \ldots
\end{aligned}
$$

Minimization by molecular dynamics

- MD solves Newton's eqns. of motion:

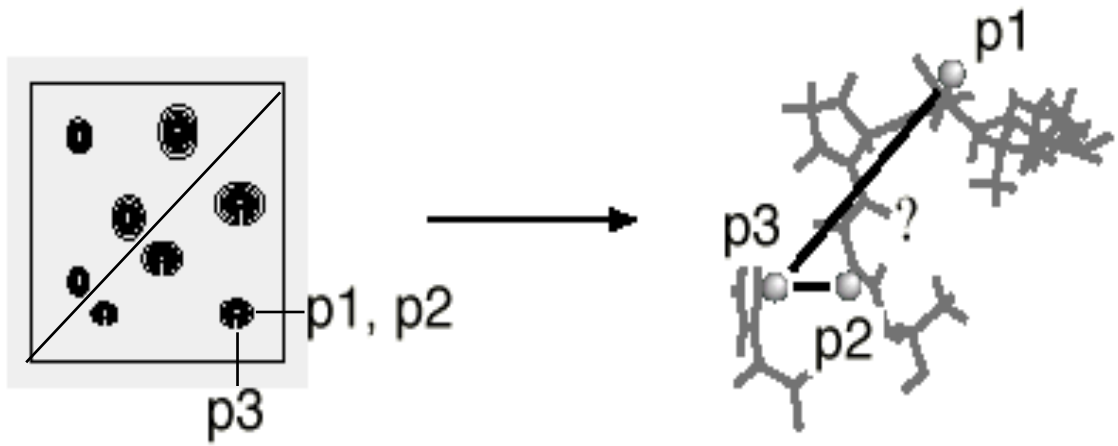
$$\frac{d^2\vec{r}_i}{dt^2} = -\frac{c}{m_i}\frac{\partial}{\vec{r}_i}E_{hybrid}$$

- Molecular dynamics can overcome local energy barriers



- Temperature control and variation: minimization by simulated annealing

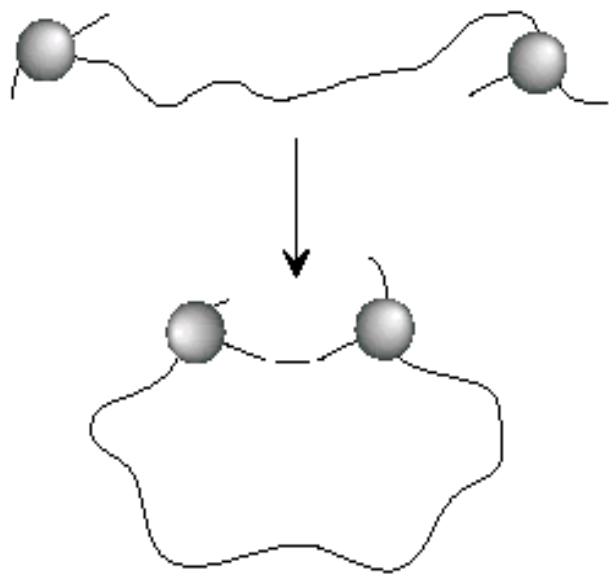
NOE ambiguities



- Key problem is ambiguity in NOE assignments
- Need for higher dimensional data: 3D & 4D
- Need for heteronuclear data
- Need for better calculational strategies that can deal with ambiguous data

Errors in data: error bounds

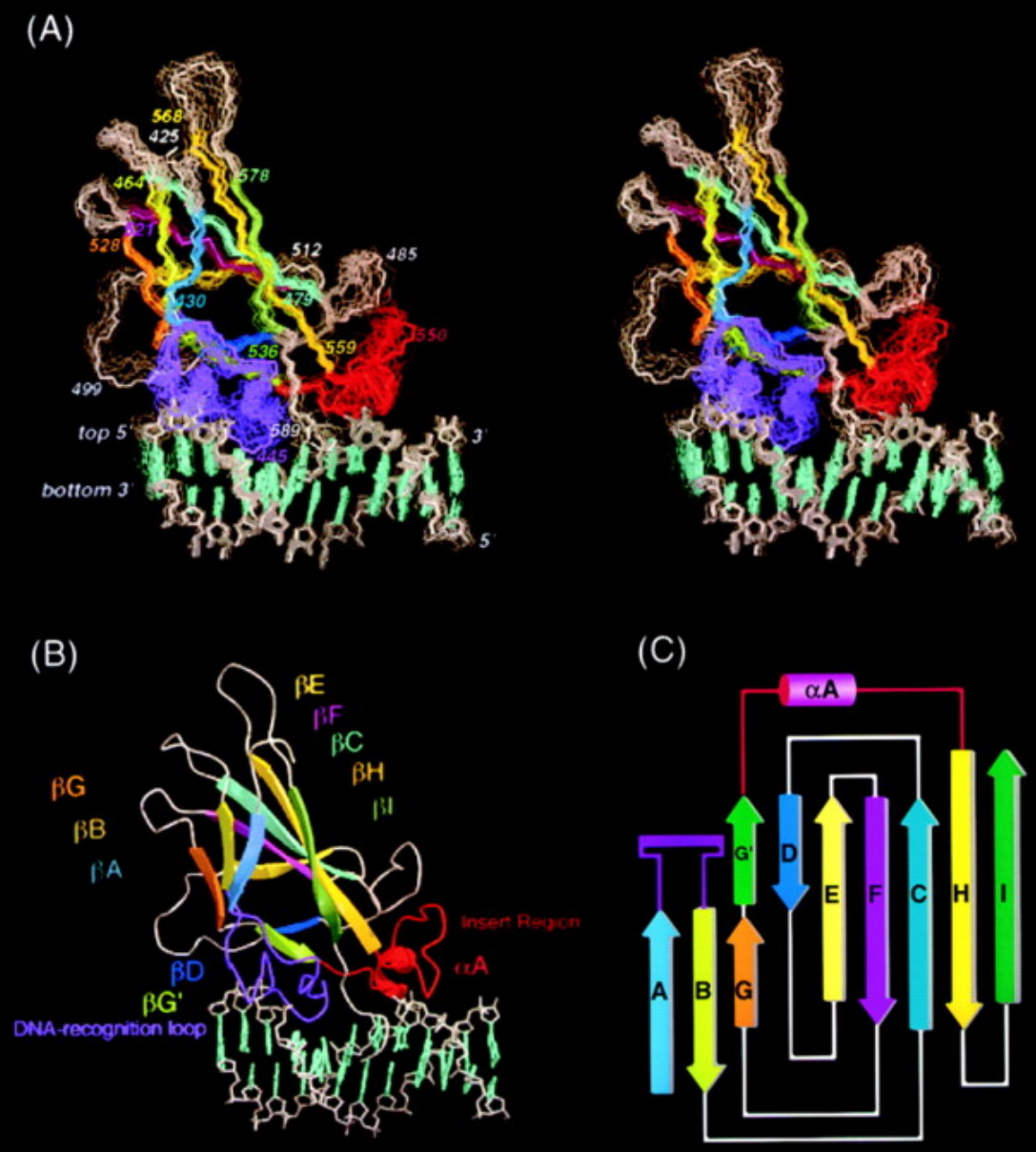
- Cumulative error in D_{ij} is treated by using loose error bounds $L \ldots U$
- Precise value not (too) critical:
  loose bounds restrict conformational space



- However, consequences for:
  - precision of structure

residues # restraints/residue

Solution Structure of the Core NFATC1/DNA Complex



Zhou et al, Cell , 1998

Need to evaluate restraint numbers, violations and precision

Table 1. Structural Statistics for NFATC1-DBD*/DNA Complex

Protein		
Amino acids residues sequentially assigned (nonproline)		162 of 167
Effective distance constraints		1050
Intraresidue		236
Sequential (|i − j| = 1)		374
Medium-range (|i − j| ≤4)		86
Long-range (|i −j| ≥5)		272
H bonds		82
Dihedral angle constraints		363
DNA		
Effective distance constraints		276
Intraresidue		146
Sequential		131
Interstrand		1
H bond		58
Protein–DNA interface		
Effective distance constraints		56
Distance constraint violations > 0.2 Å (per structure)		1.81 ± 2.07
Dihedral constraint violations > 3.0° (per structure)		1.44 ± 0.96
X-PLOR potential energy (E_{L-J}, Kcal/mol, avg. per structure)		−501 ± 28.9
R.m.s.d. to the mean for backbone heavy atoms of all β strands (residues 428–432, 460–464, 472–479, 493–495, 506–510, 515–521, 528–538, 559–568, 578–583)		0.62 ± 0.07
R.m.s.d. to the mean for heavy atoms of all β strands (residues 428–432, 460–464, 472–479, 493–495, 506–510, 515–521, 528–538, 559–568, 578–583)		1.20 ± 0.14
R.m.s.d. to the mean for backbone heavy atoms (residues 425–590)		1.14 ± 0.11
R.m.s.d. to the mean for heavy atoms (residues 425–590)		1.58 ± 0.10
R.m.s.d. to the mean for DNA heavy atoms (superposition of all DNA heavy atoms)		0.83 ± 0.21
R.m.s.d. to the mean for protein + DNA heavy atoms (superposition of all β strand heavy atoms and DNA heavy atoms)		1.20 ± 0.16
Ramachandran plot[a]		
	Residues 425–590	Secondary Structures
Most favorable region	60.4%	83.7%
Additionally allowed region	38.9%	14.0%
Generously allowed region	0.7%	2.3%
Disallowed region	0.0%	0.0%

[a] Laskowski et al., 1996.

How were restraints measured?

Analysis of Table

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How many NOE restraints?

>6 per residue is acceptable

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How are NOE restraints distributed?

Reading the NMR Statistics Structure Table

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Distance constraint violations > 0.2 Å (per structure)		1.81 ± 2.07
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X-PLOR potential energy (E_{L-J}, Kcal/mol, avg. per structure)		−501 ± 28.9
R.m.s.d. to the mean for backbone heavy atoms of all β strands (residues 428–432, 460–464, 472–479, 493–495, 506–510, 515–521, 528–538, 559–568, 578–583)		0.62 ± 0.07
R.m.s.d. to the mean for heavy atoms of all β strands (residues 428–432, 460–464, 472–479, 493–495, 506–510, 515–521, 528–538, 559–568, 578–583)		1.20 ± 0.14
R.m.s.d. to the mean for backbone heavy atoms (residues 425–590)		1.14 ± 0.11
R.m.s.d. to the mean for heavy atoms (residues 425–590)		1.58 ± 0.10
R.m.s.d. to the mean for DNA heavy atoms (superposition of all DNA heavy atoms)		0.83 ± 0.21
R.m.s.d. to the mean for protein + DNA heavy atoms (superposition of all β strand heavy atoms and DNA heavy atoms)		1.20 ± 0.16
Ramachandran plot[a]		
	Residues 425–590	Secondary Structures
Most favorable region	60.4%	83.7%
Additionally allowed region	38.9%	14.0%
Generously allowed region	0.7%	2.3%
Disallowed region	0.0%	0.0%

[a] Laskowski et al., 1996.

How were H Bonds Determined?

Reading the NMR Statistics Structure Table

Table 1. Structural Statistics for NFATC1-DBD*/DNA Complex

Protein		
Amino acids residues sequentially assigned (nonproline)		162 of 167
Effective distance constraints		1050
Intraresidue		236
Sequential (|i − j| = 1)		374
Medium-range (|i − j| ≤4)		86
Long-range (|i −j| ≥5)		272
H bonds		82
Dihedral angle constraints		363
DNA		
Effective distance constraints		276
Intraresidue		146
Sequential		131
Interstrand		1
H bond		58
Protein–DNA interface		
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How many intermolecular NOEs?

Reading the NMR Statistics Structure Table

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[a] Laskowski et al., 1996.

Criteria for publication:
No distance violations >0.5 Å
No dihedral angle violations >5°

How many violations ?

Read methods section to evaluate table

Resonance Assignments

1-HNCA/HN(CO)CA
2-Amino-acid specific labeling
3-NOESY experiments:
Homonuclear and heteronuclear

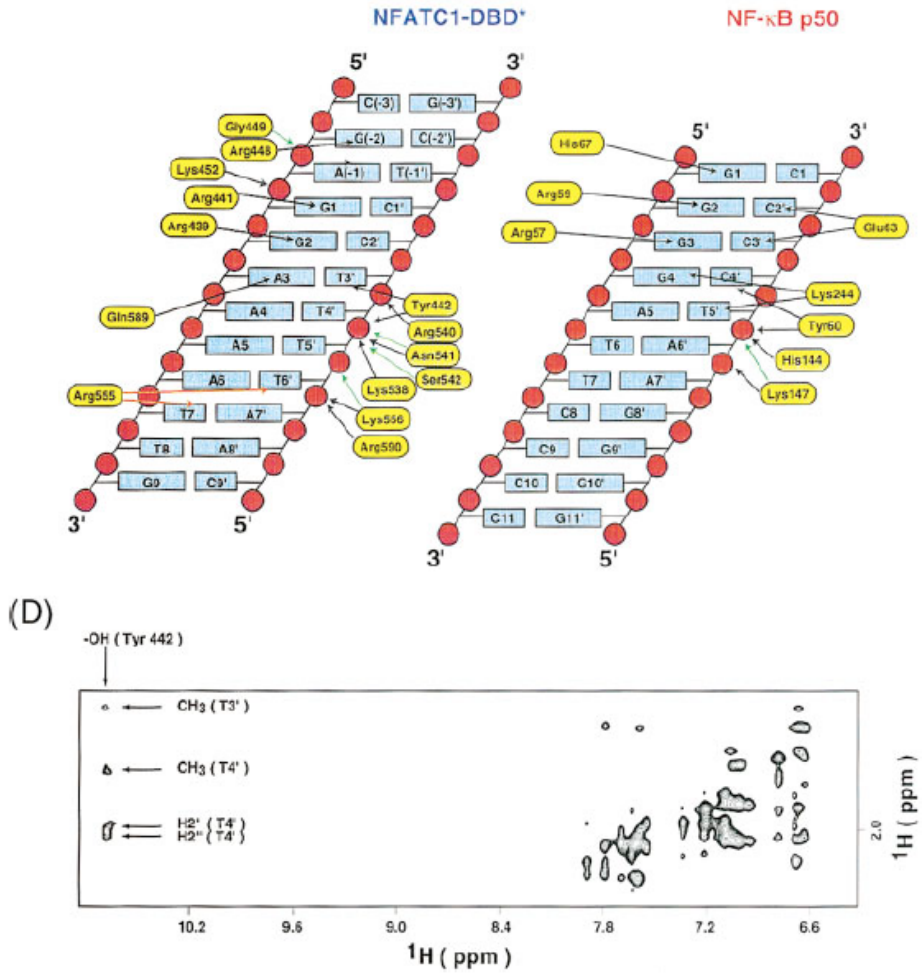
Distance Restraints

1-2D and 3D NOESY at longer mixing times to get weak NOEs
2-HD exchange to get hydrogen bonds

Many of these experiments performed with a specific labeling scheme to facilitate NOEs assignment

Side chain assignments from NOESY, caveats?

Assymetric isotope labelling to get intermolecular NOEs



Protein is deuterated, DNA protonated experiment done in D2O solvent

Caveats?

Structures of larger proteins and complexes

Sample Deuteration Increases Sensitivity and Resolution

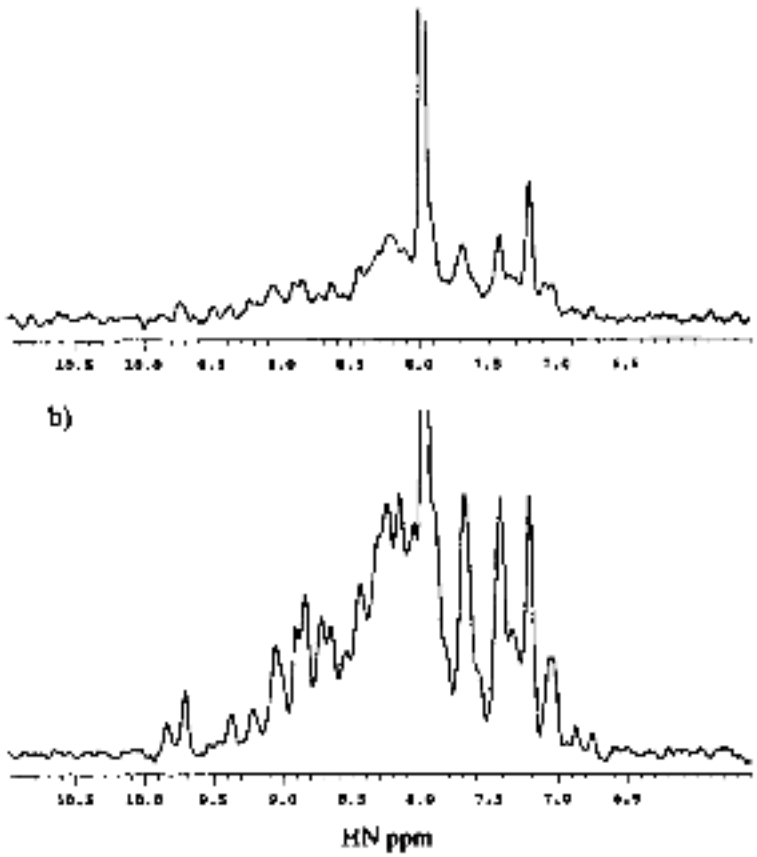
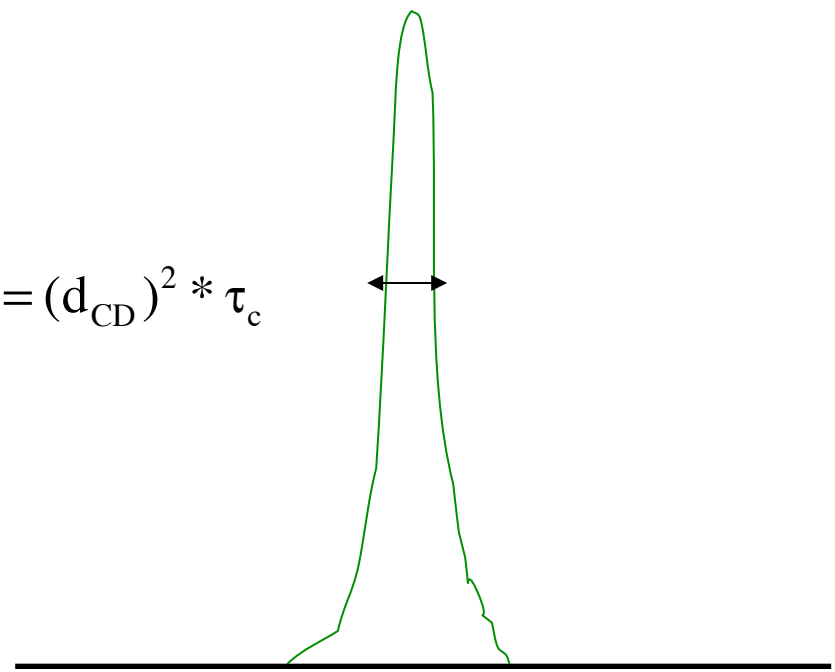


Figure 14. Comparative 1D spectra of fully protonated (a) and ~70% deuterated (b) ^{15}N, ^{13}C trpR using the HN(CA)CB scheme (Figure 2c). A total of 1024 transients were recorded for the ^{2}H sample and 6928 transients (1024×2.6^2) for the ^{1}H sample which is a factor of 2.6 more dilute. The rms noise levels are normalized in the plots.

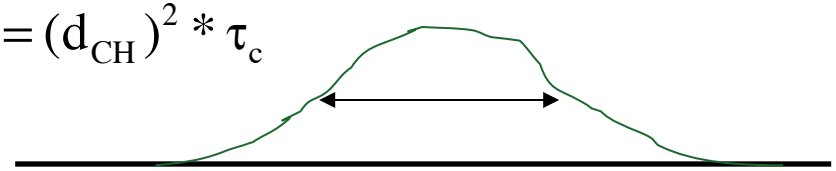
Why does deuteration help?

$$d_{CH} = \frac{\gamma_C \gamma_H}{r^3} \frac{3\mathrm{Cos}^2(\theta) - 1}{2}$$

$$\frac{1}{T_2} = (d_{CD})^2 * \tau_c$$



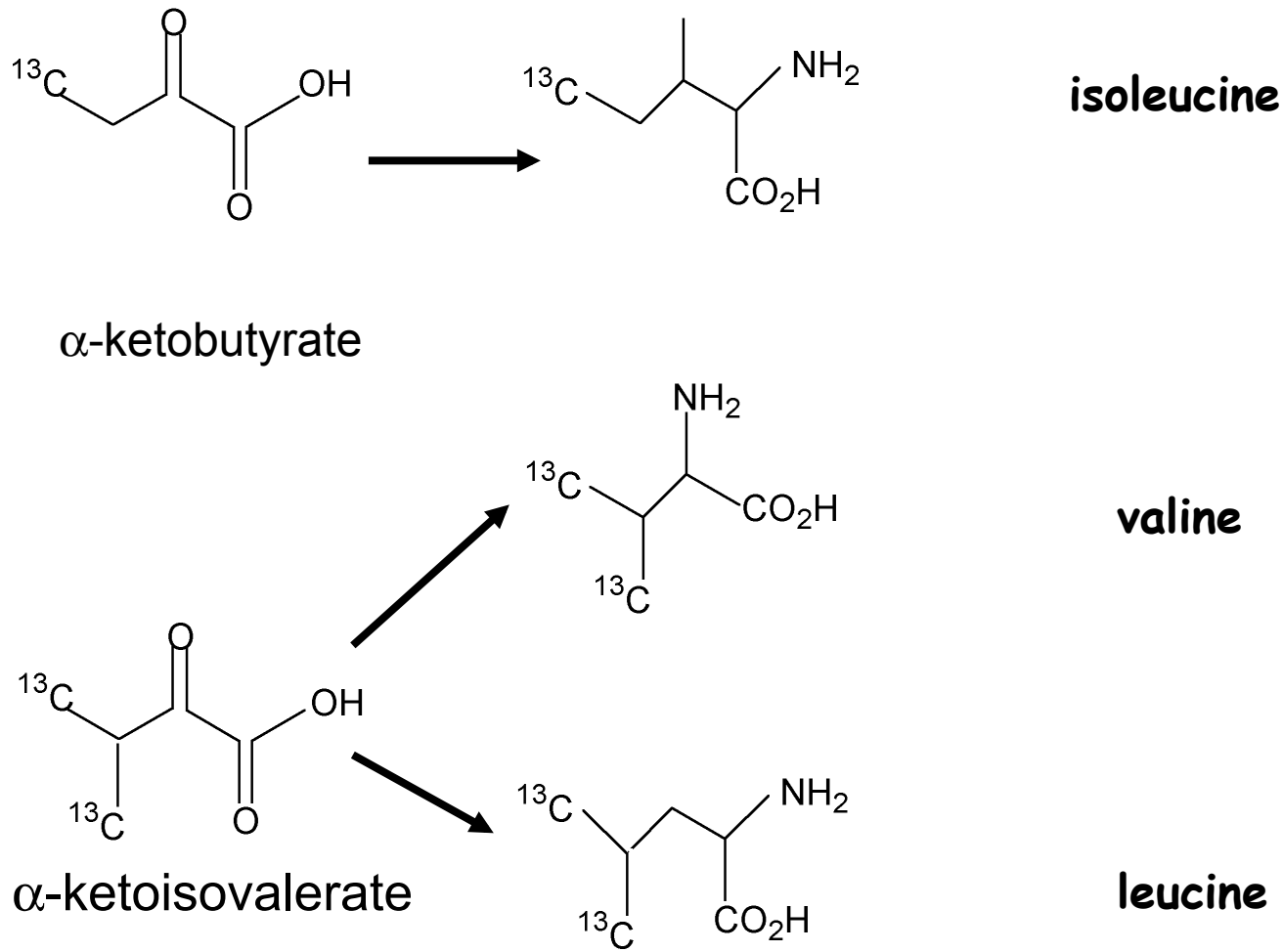
$$\frac{1}{T_2} = (d_{CH})^2 * \tau_c$$



Dipolar coupling for CH spin pair 6.5 times stronger than for CD--->roughly 50 fold reduction in linewidth with increase in S:N.

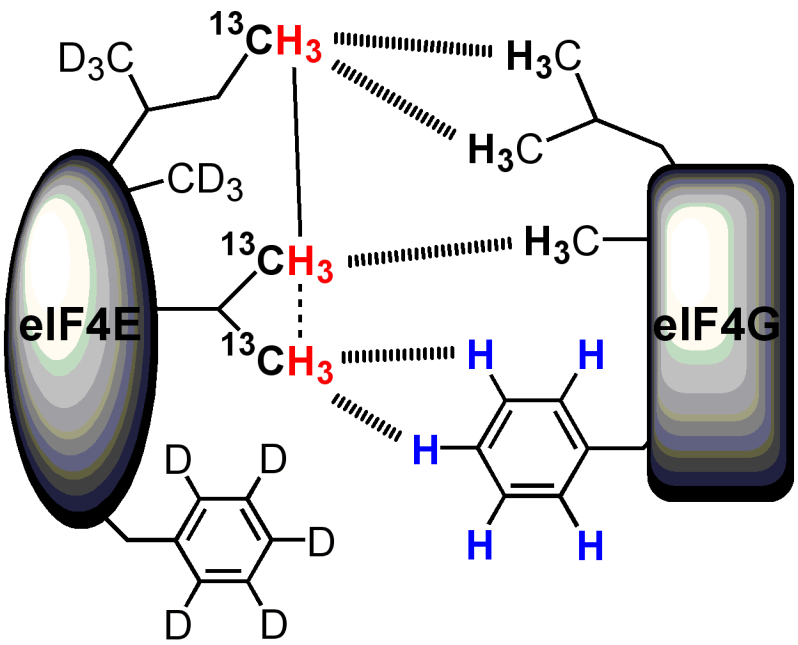
Need methods for measuring distance restraints in sparse ^{1}H environment

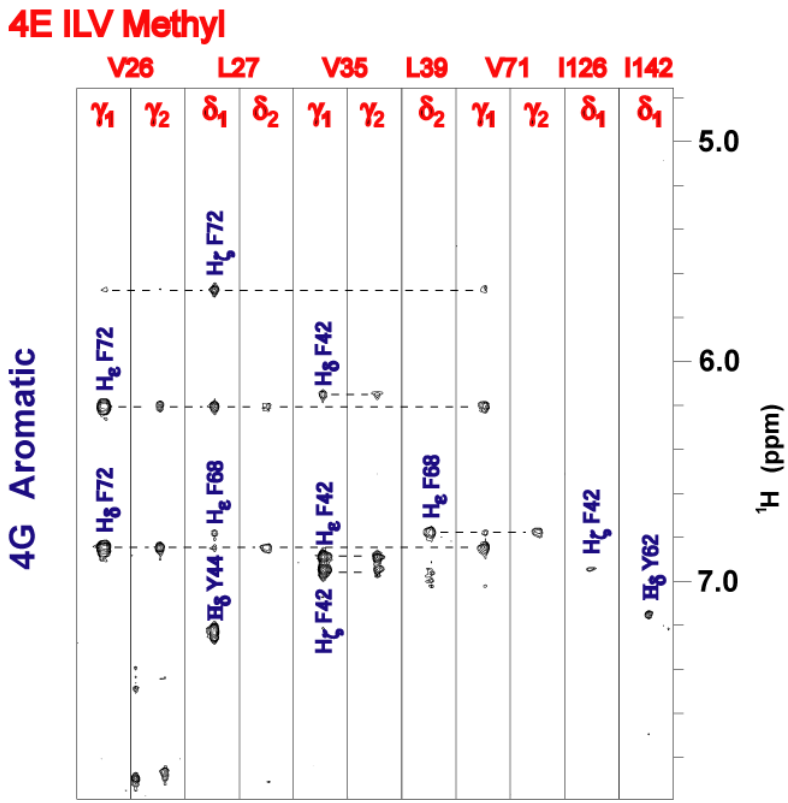
Selective Re-introduction of Protons for NOE experiments



Grow E. coli in D2O and deuterated glucose, add precursors to introduce 1H/13C methyl labels

Measurement of Intermolecular NOEs using Asymmetric Deuteration with ILV Labeling

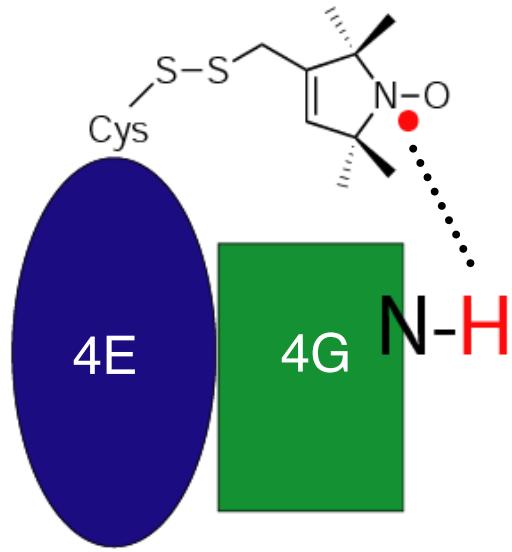




Aromatic/methyl NOEs are unambiguously identified

Gross , Gelev and Wagner, J Biomol NMR 2003

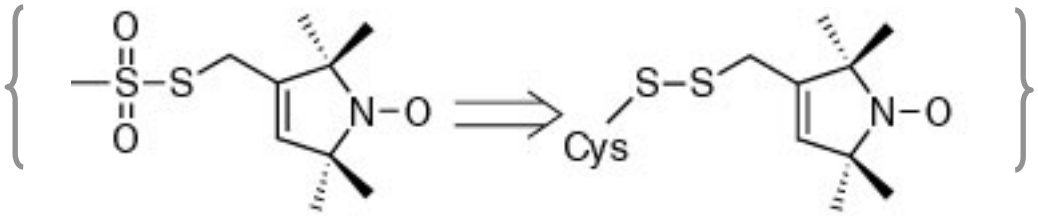
Determining Long Range Distances through Paramagnetic Relaxation Enhancements



Dipolar broadening between unpaired electron and ^{1}H: $1/r^{6}$ dependence.

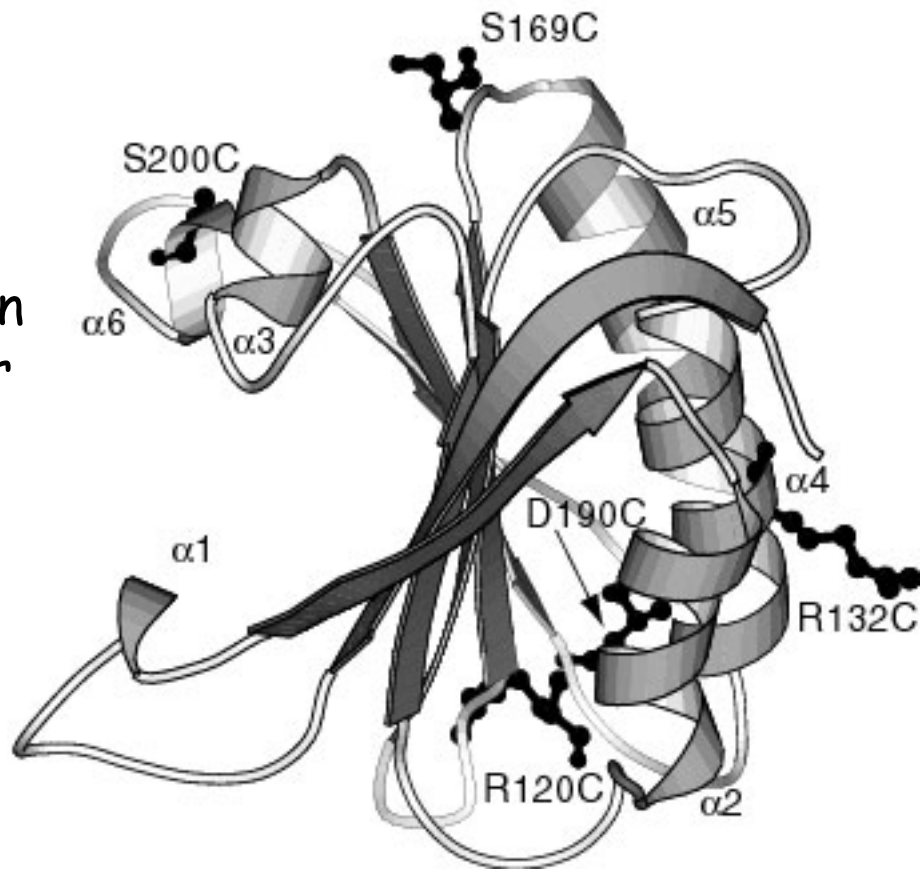
Provides long range distance information (15-20 angstroms)

Site-Directed Spin Labeling for PRE



MTSL-Cysteine Adduct

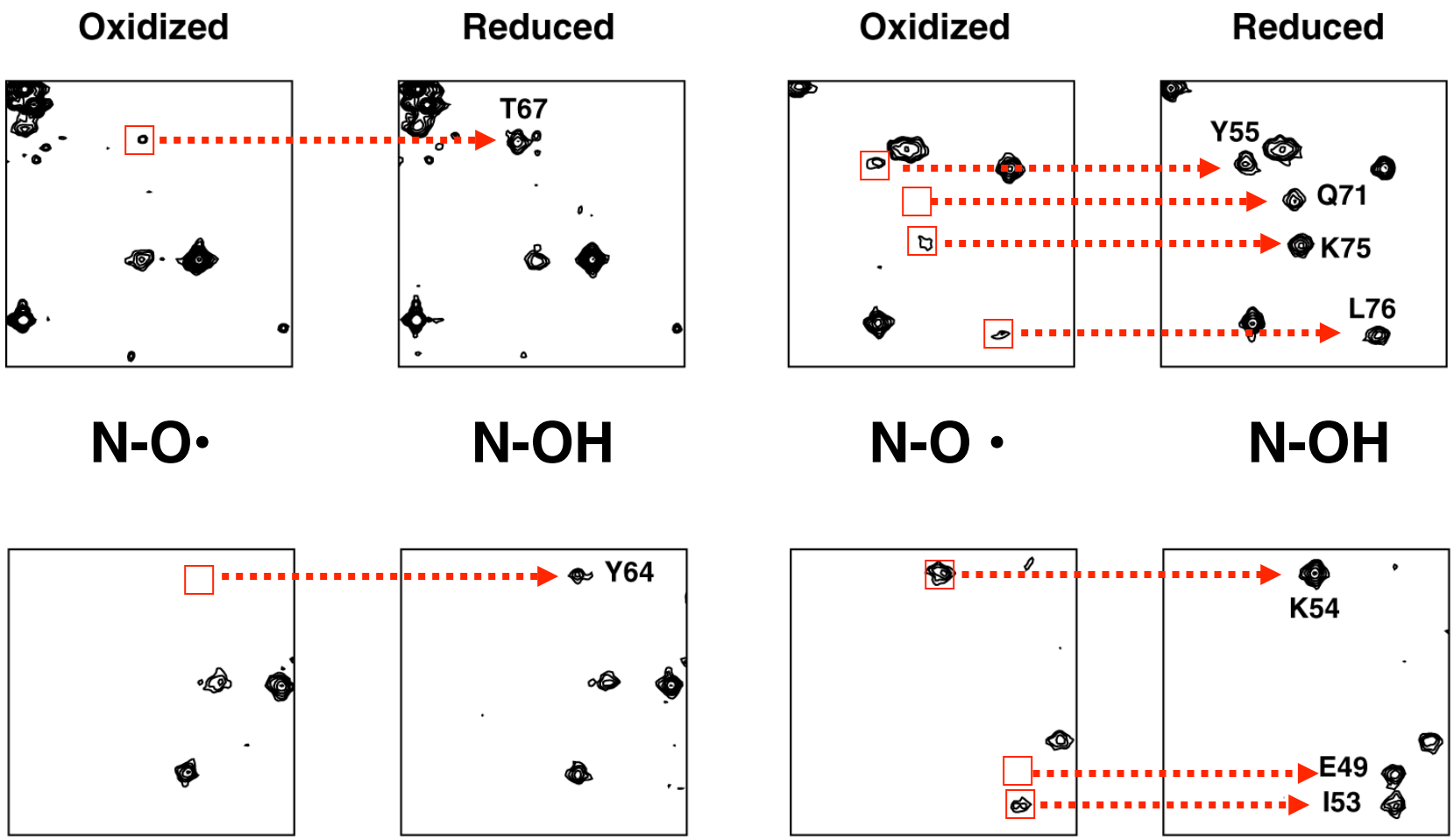
Cysteine mutation in surface loop or helix



Record HSQC In absence and presence of reductant

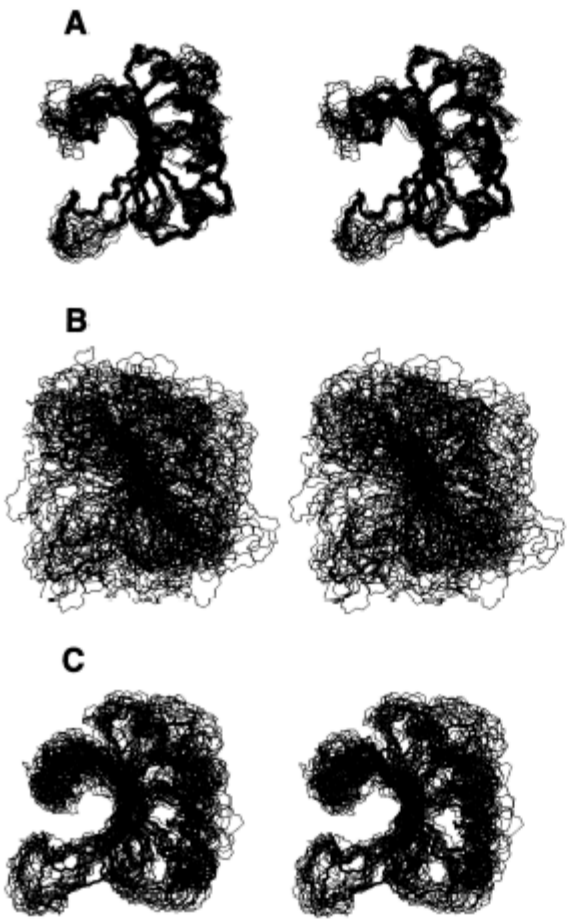
Battiste and Wagner , Biochemistry, 2000

Paramagnet Relaxation Enhancement from Site-Directed Spin Labelling



Intensity reduction from dipolar coupling ($1/r^6$), so distances can be extracted

Impact of PRE restraints for structure determination



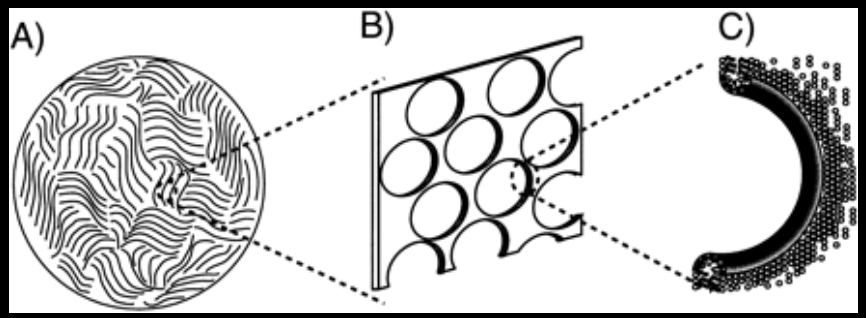
ALL NOEsm (2014)

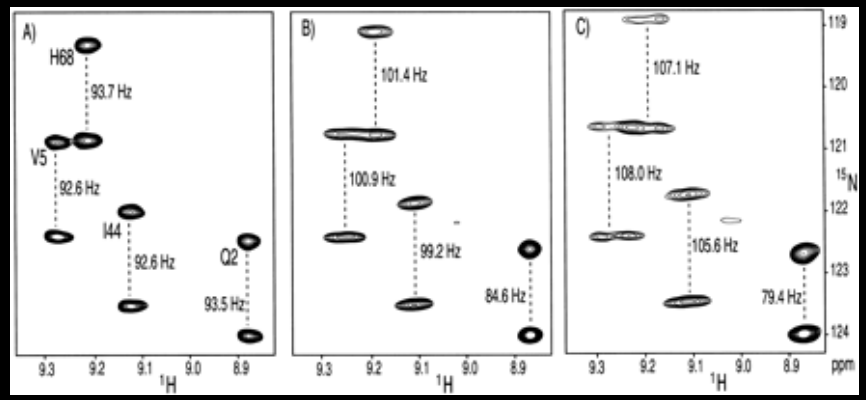
ONLY HN-HN NOEs (403)

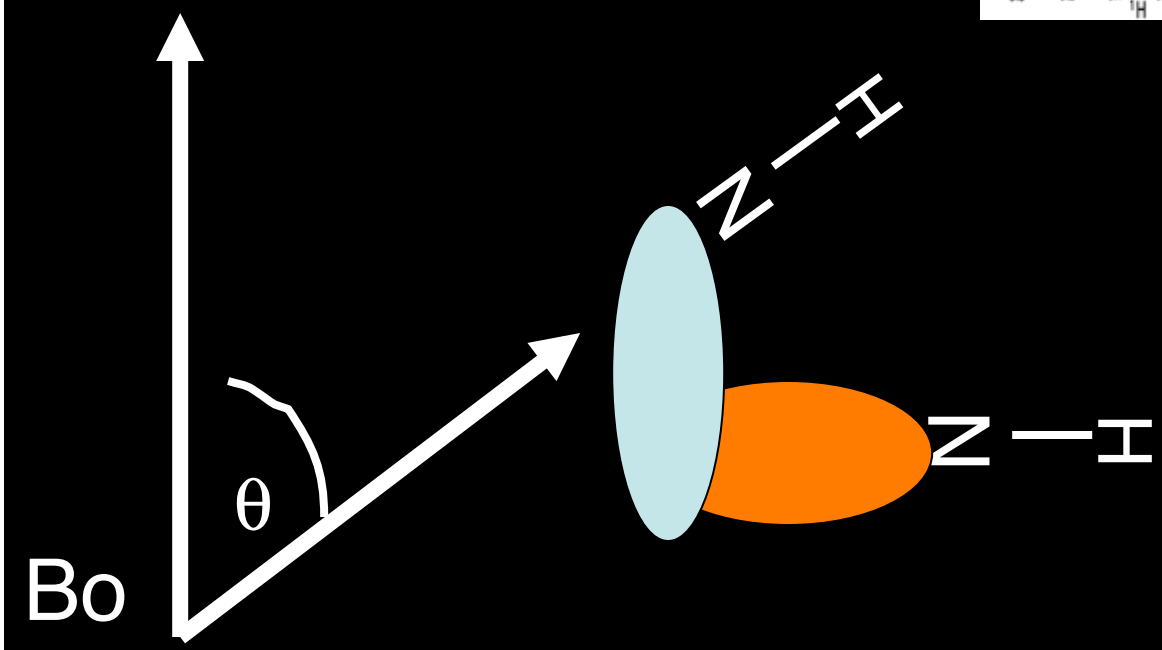
HN-HN NOEs + PRE (515)

Battiste and Wagner , Biochemistry, 2000

Residual Dipolar Couplings



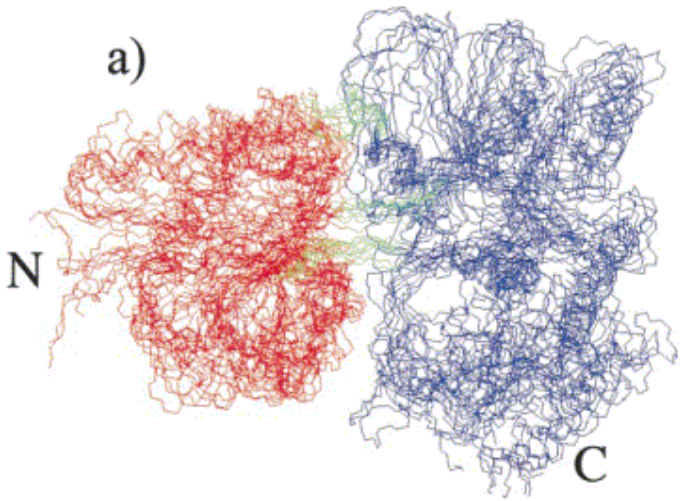


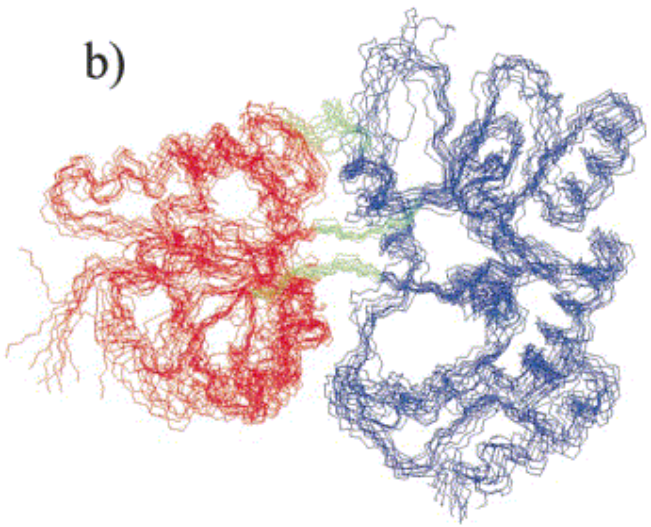


$$d_{NH}\,[3\cos^2\theta - 1]/2$$

$$d_{NH} \sim \frac{\gamma_C \gamma_H}{r^3}$$

Impact of RDCs on Precision and Accuracy: MBP, a 42 kDa test Case





RMSD

Precision:	5.5Å	2.2 Å
Accuracy:	5.1Å	3.3 Å

From Mueller et. Al. JMB 300(1) 197-212 2000

Prospects for even Larger Proteins

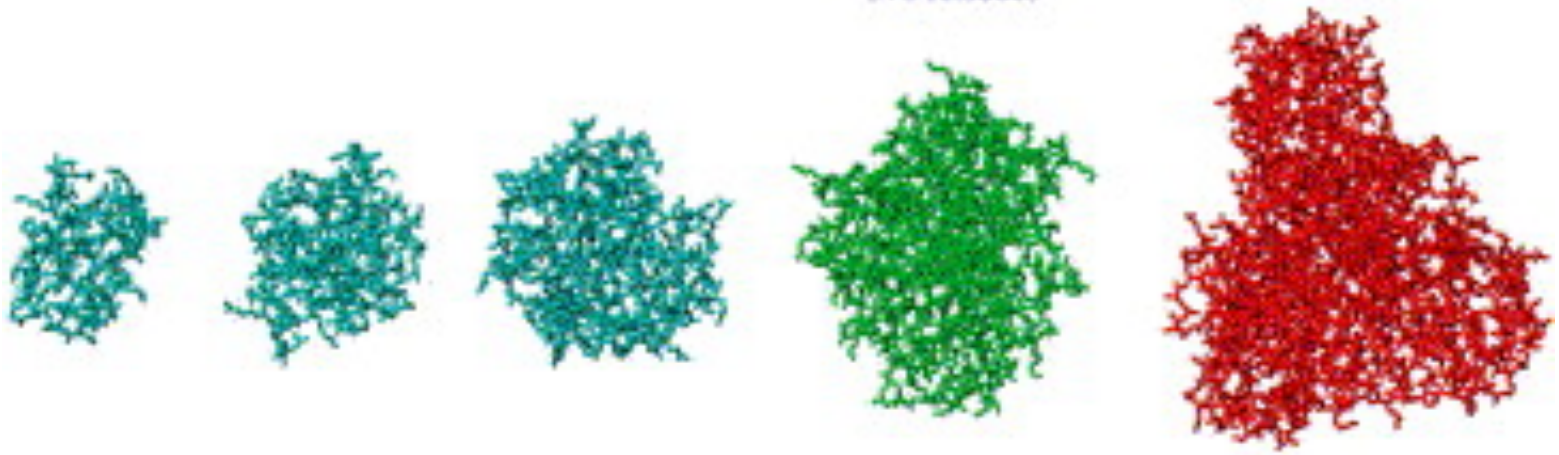
Ubiquitin
76 residues

Lysozyme
156 residues

Adenylate Kinase
214 residues

Maltose binding
protein (MBP)
370 residues

Malate synthase G
723 residues



i. NMR assignments
ii. Structure determination
iii. Dynamics

requires ^{15}N, ^{13}C labeling

i. NMR assignments
ii. Global fold/structure determination
iii. Domain orientation
iv. Dynamics

requires ^{15}N, ^{13}C, ^{2}H labeling

?

X-ray	vs	NMR
• crystal • single structure-best fit to electron density		• solution • ensemble of 20-50 structures that equally fit experimental data

Limitations of NMR

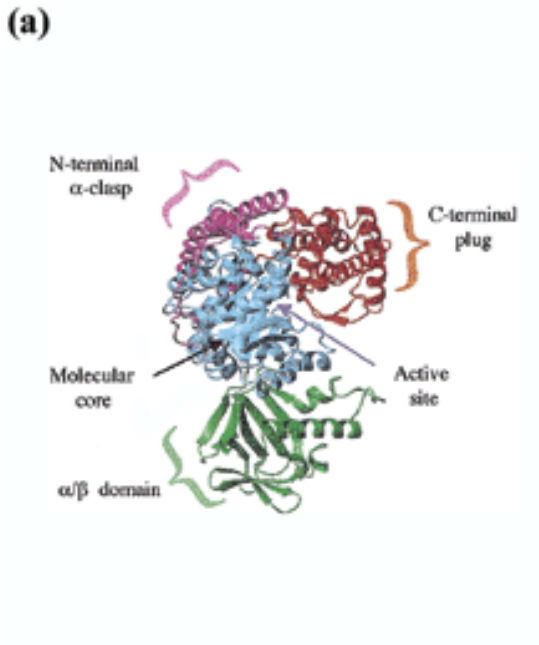
- small proteins (20-30 kD max, although this is changing)
- must be soluble and nonaggregating at 1-3 mM conc
- lots of protein needed

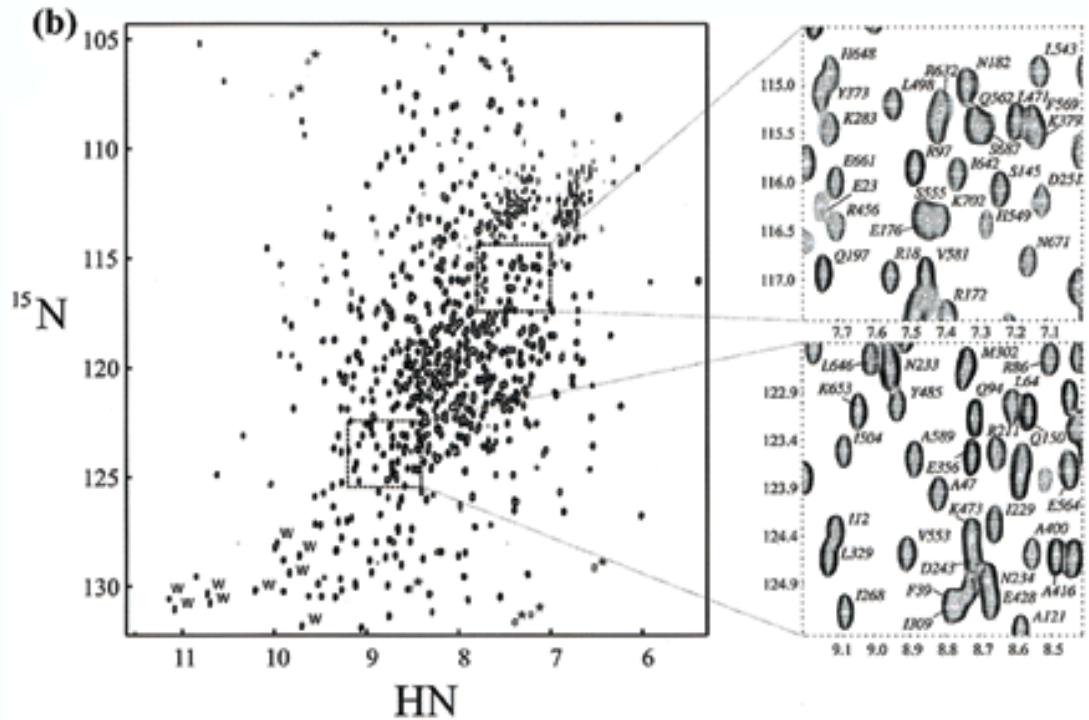
Advantages of NMR

- don't need crystal
- observe protein in solution
- more than a method for determining structure

dynamics
ligand binding (drug/protein/DNA/etc)
protein folding
conformational change
chemistry, chemical reactions, protonation states.....

TROSY



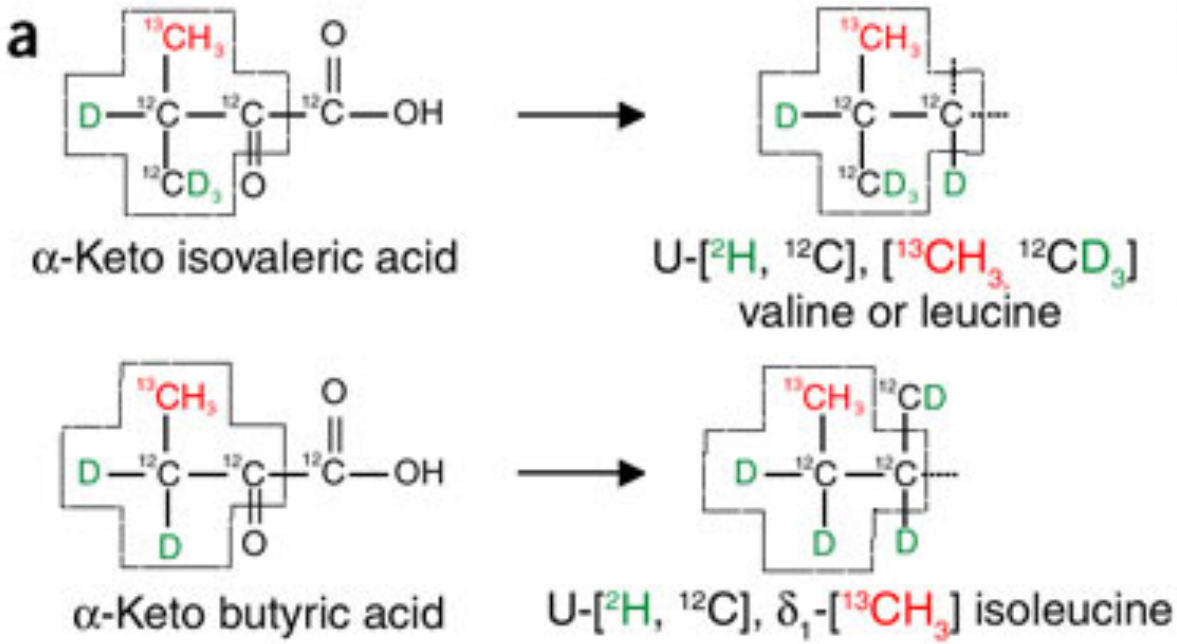


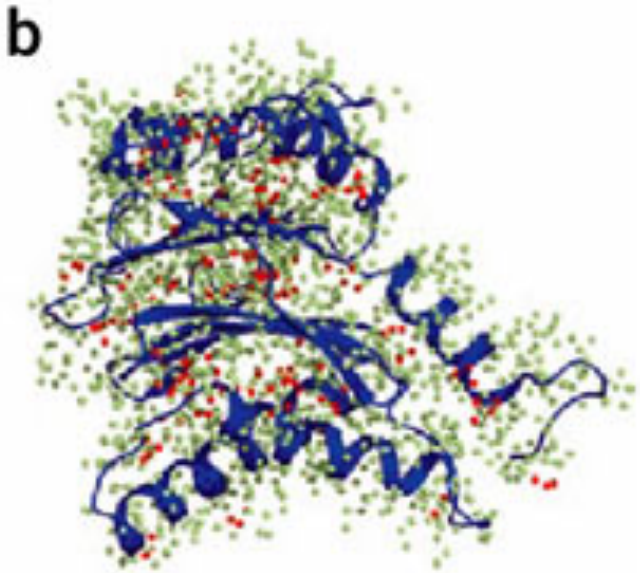
Malate Synthase G
80 kDa

Turgarinov et al, JACS 2002

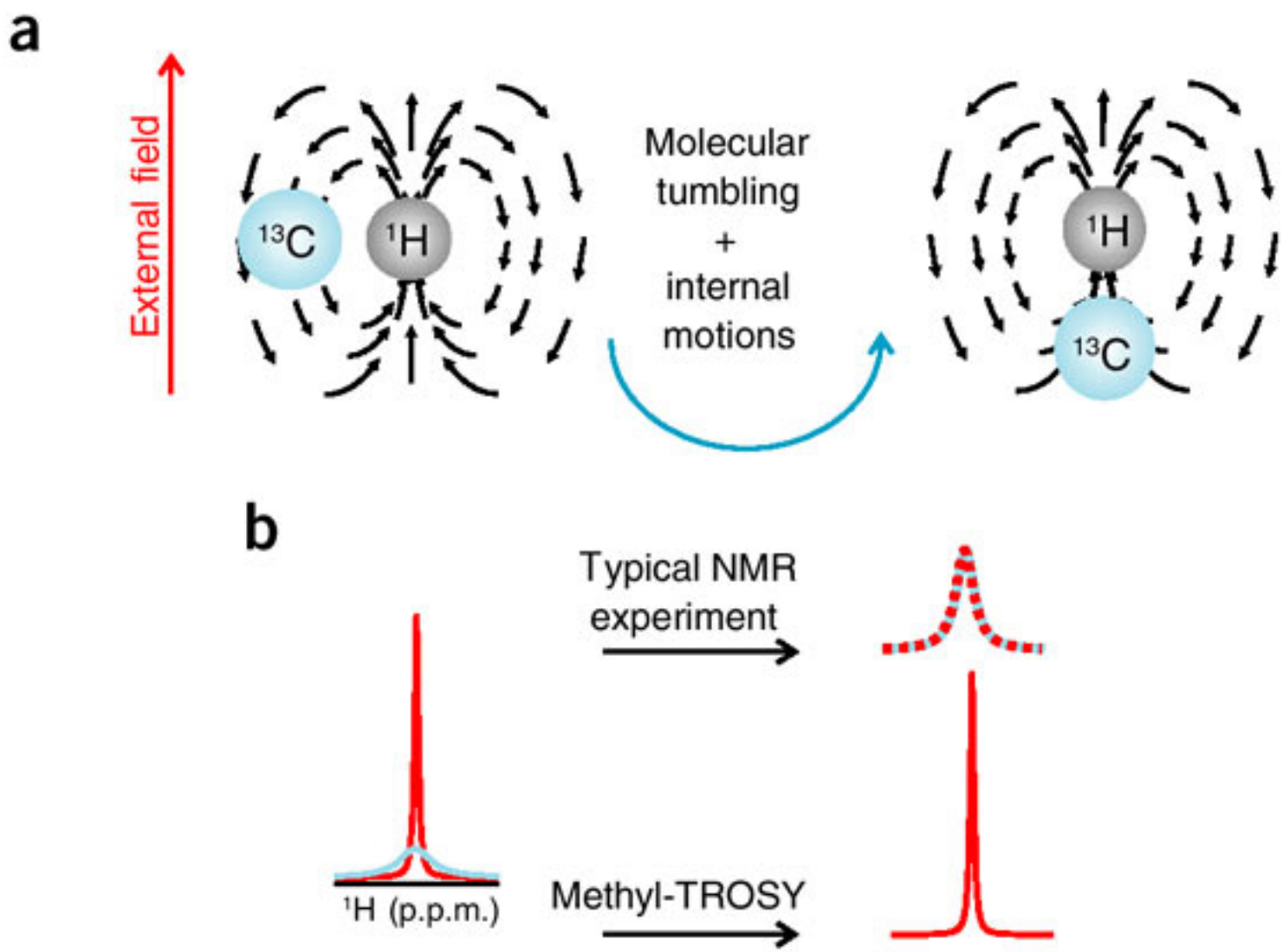
Same info as ^{15}N HSQC

Methyl-group labeling





Methyl-TROSY



doi:10.1038/nature05512

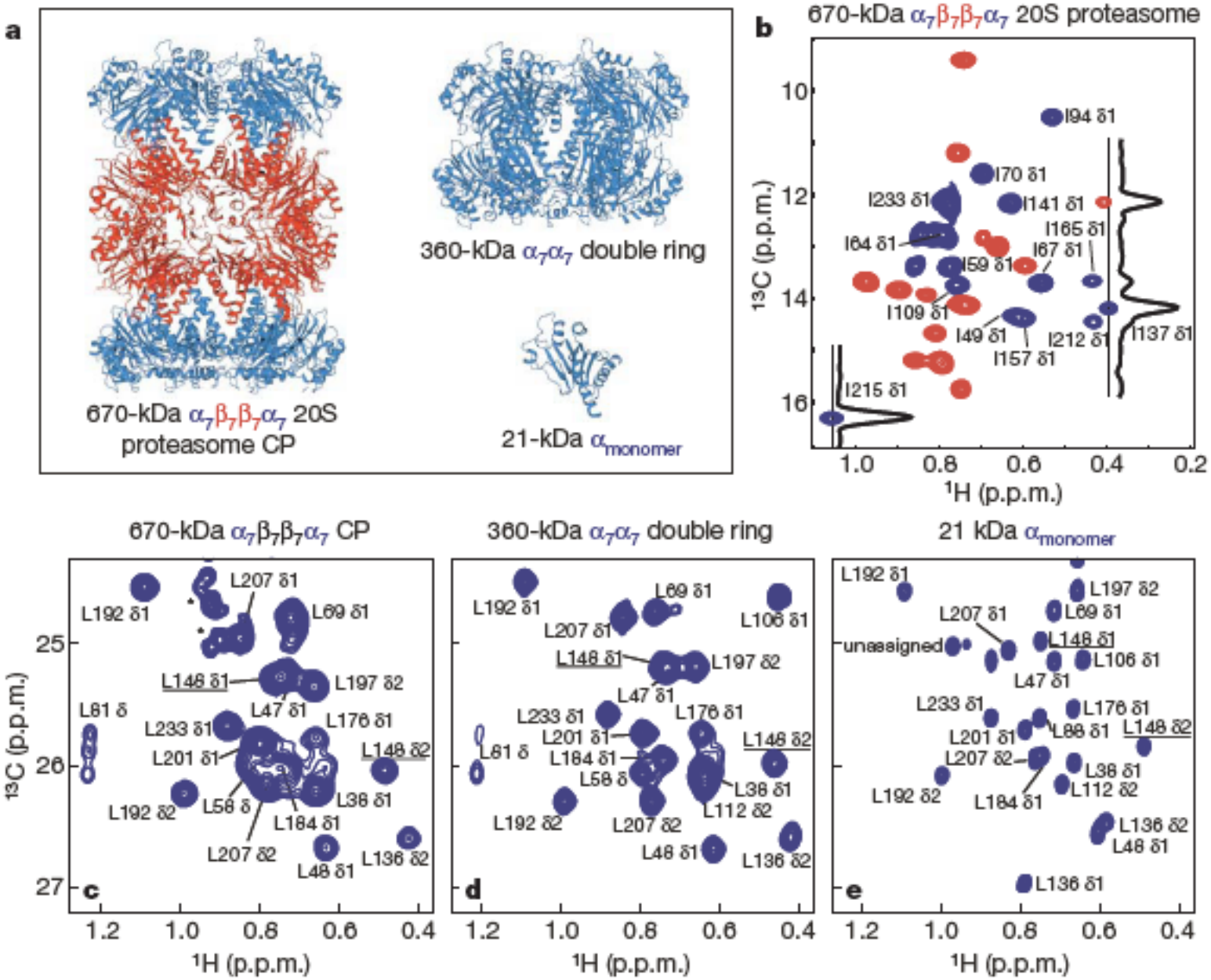
nature

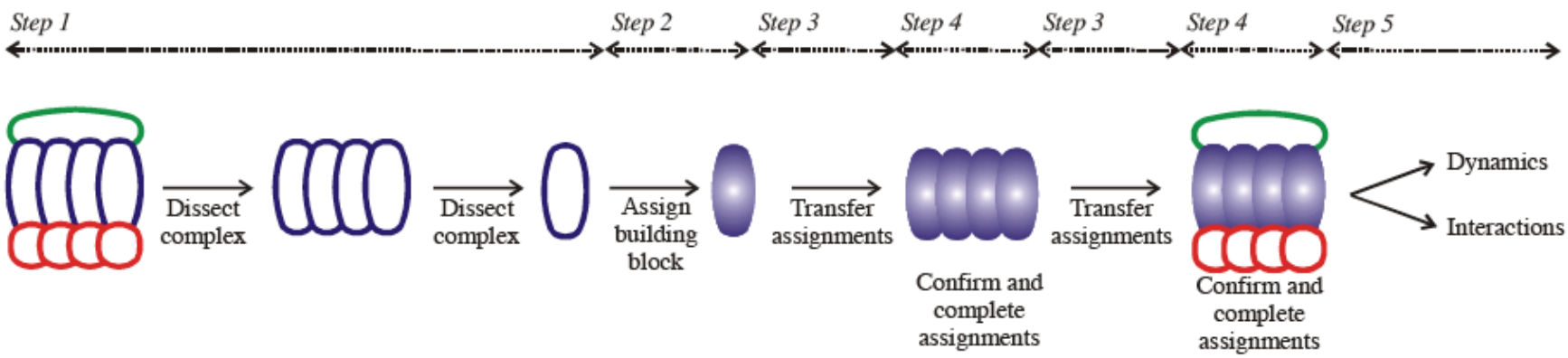
ARTICLES

Quantitative dynamics and binding studies of the 20S proteasome by NMR

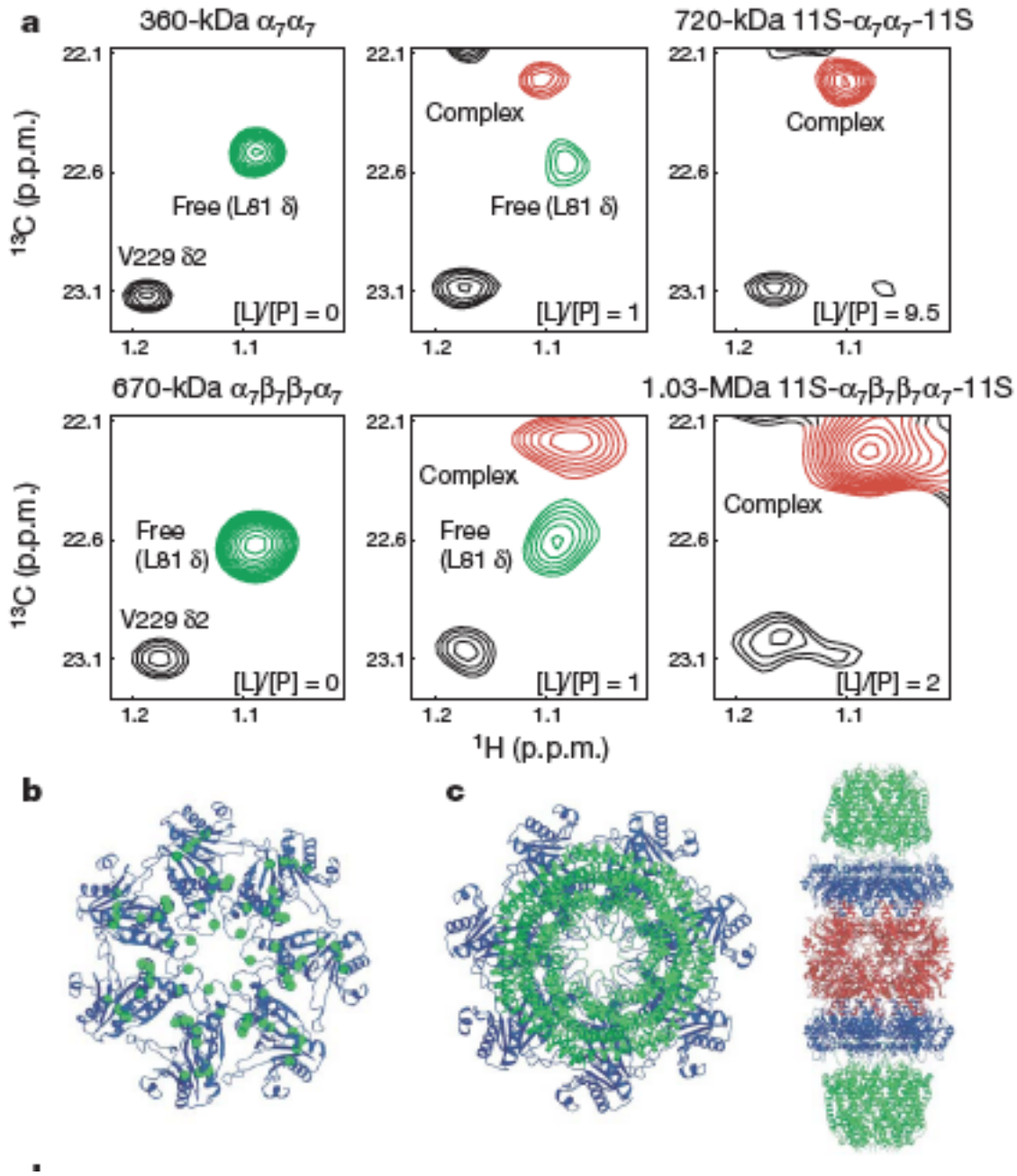
Remco Sprangers[1] & Lewis E. Kay[1]

ILV METHYL ASSIGNMENTS OF 670 KDA COMPLEX

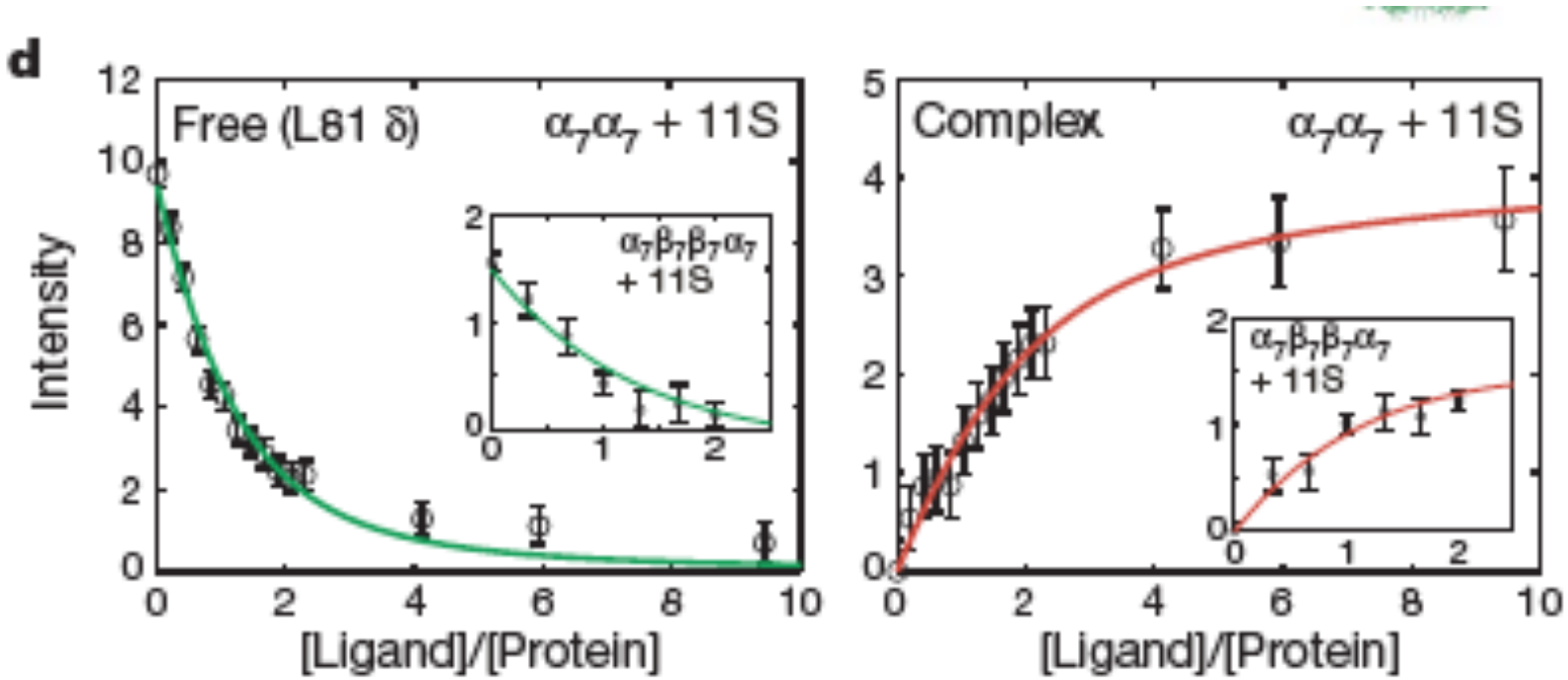



Step 1
Step 2
Step 3
Step 4
Step 3
Step 4
Step 5
Dissect complex
Dissect complex
Assign building block
Transfer assignments
Confirm and complete assignments
Transfer assignments
Confirm and complete assignments
Dynamics
Interactions

11S ACTIVATOR BINDING



11S BINDING CURVES



Applications for NMR

- Mapping protein interactions
- Fragment based drug discovery, SAR-by-NMR
- Structure of macromolecules (≤ 40KDa is practical limit)
- TROSY , deuteration and ILV labeling for large systems
- Protein folding
- Protein dynamics