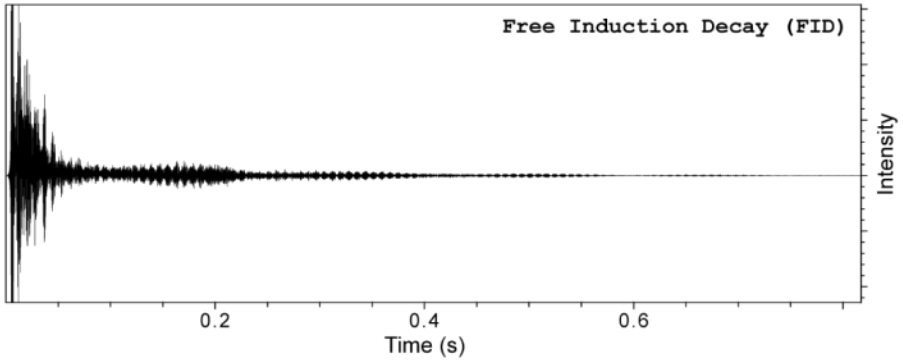
What is an assignment?

Associate a given signal back to the originating spin

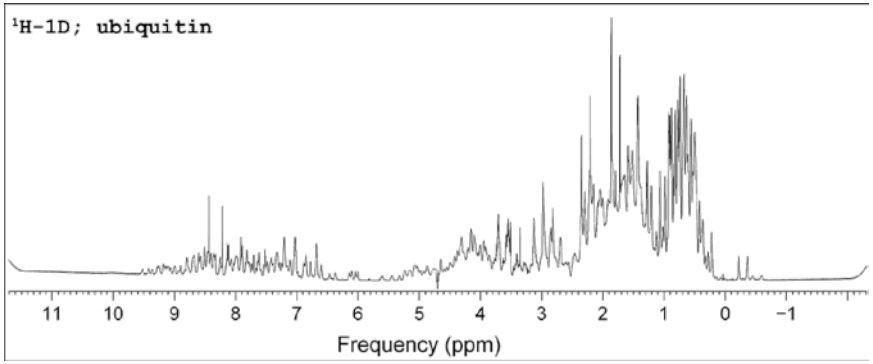
Every atom with spin has a Larmor frequency

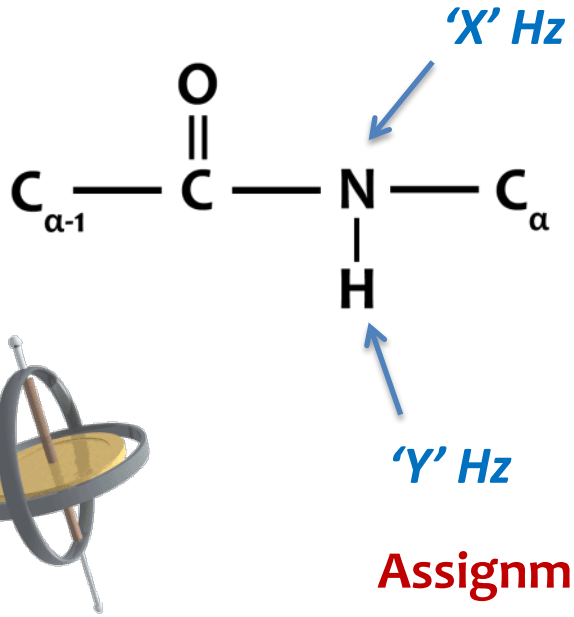


Fourier Transform (FT)

Time → Frequency

$$\delta\ (ppm) = \frac{(\nu_S - \nu_R)}{\nu_R} \times 10^6$$

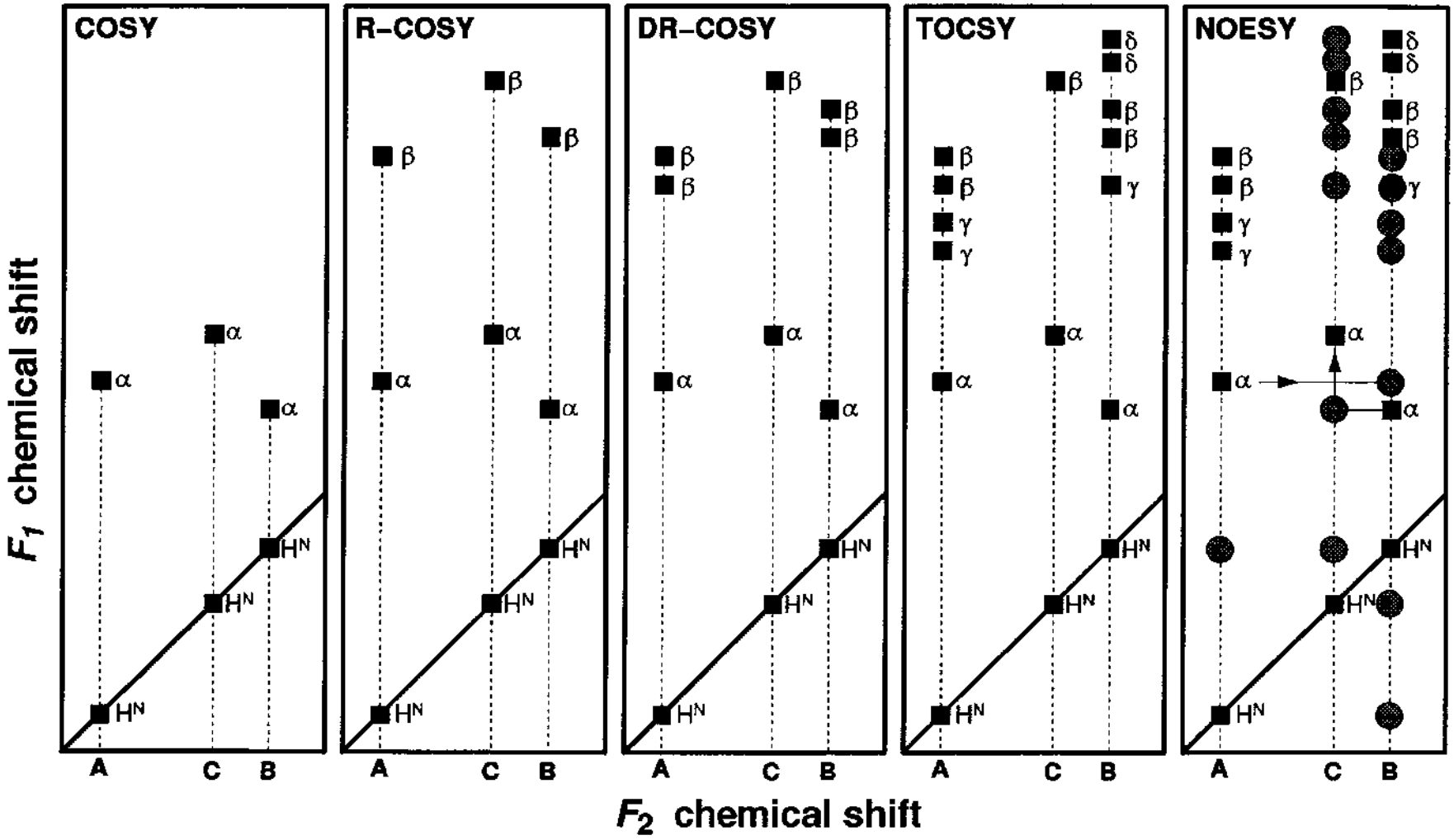




Assignments!

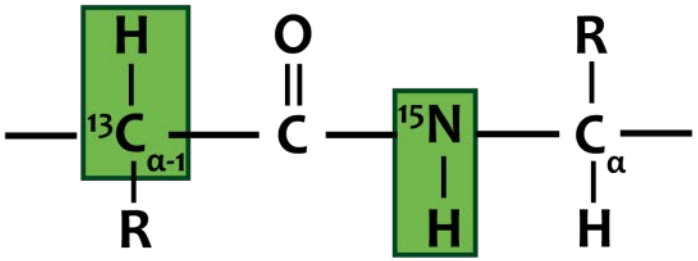
This frequency is a physical property of that atom – it is the same value no matter what experiment is run!

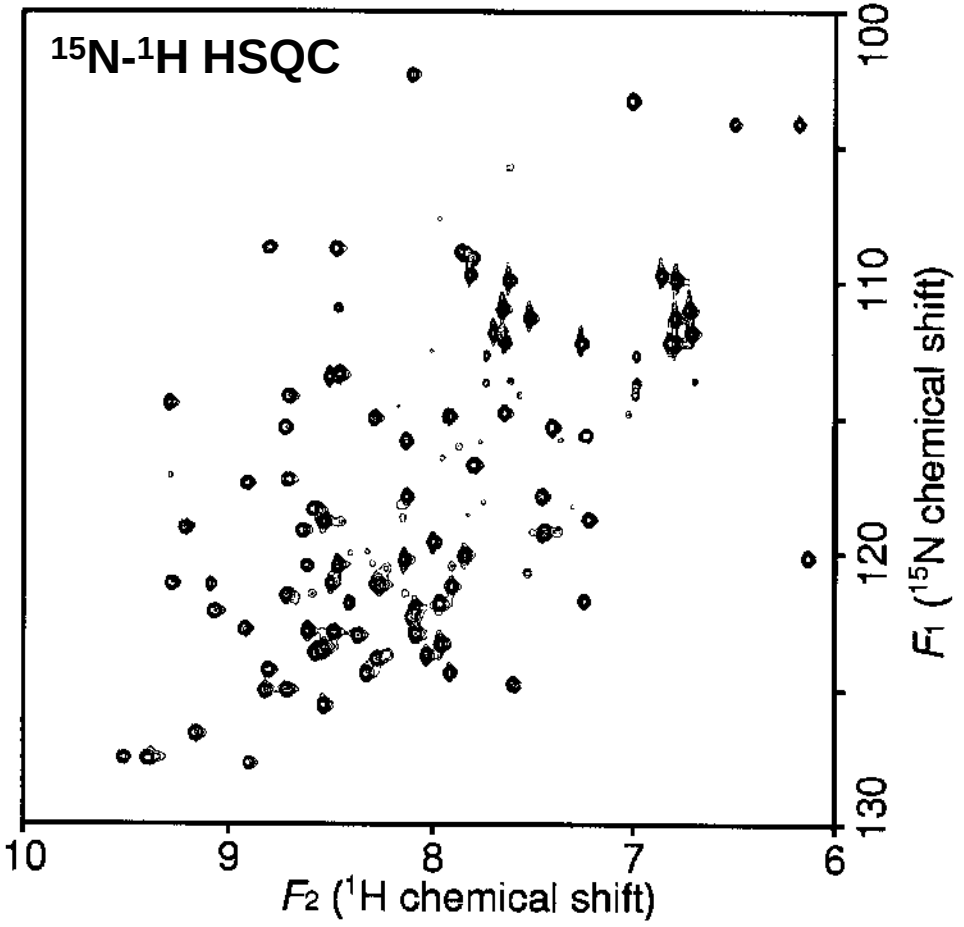
Homonuclear 2D Expts



Double-Resonance Experiments

Increases Resolution/Information Content





Basic Strategy to Assign Resonances

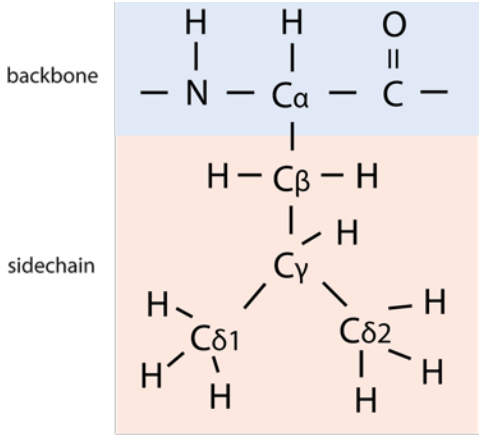
1. **Identify resonances for each residue (scalar)**

T G L S S R G

2. **Put residues in order**

1 2 3 4 5 6 7

R - G - S - T - L - G - S



Leucine (L)

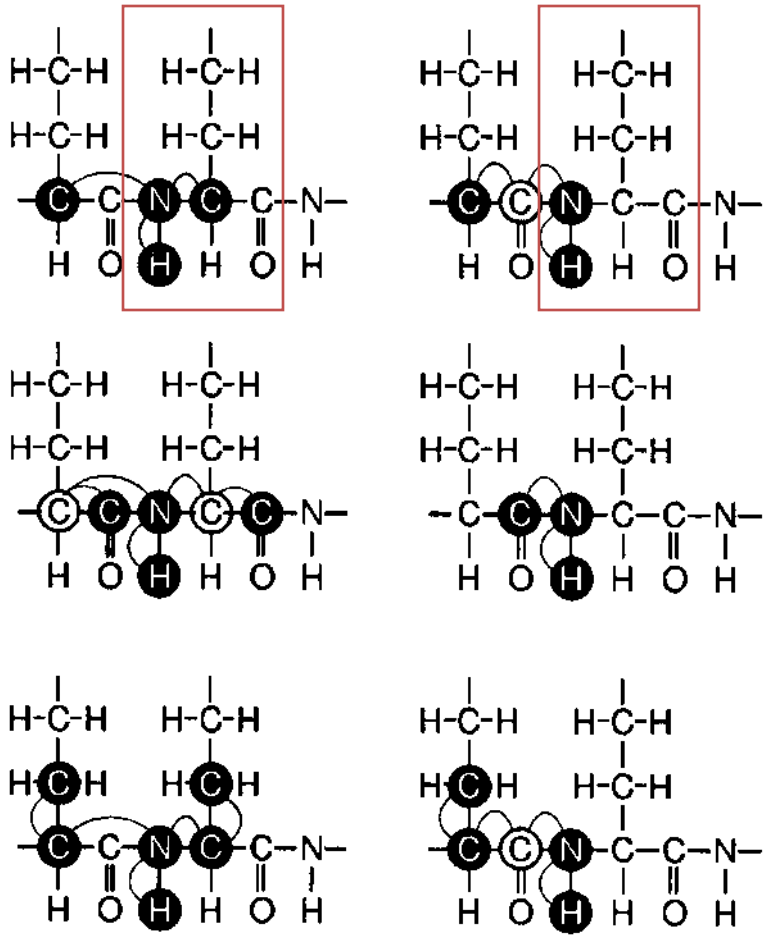
Heteronuclear Triple-Resonance Backbone Experiments

Names of scalar experiments based on atoms detected

HNCA / HN(CO)CA

HN(CA)CO / HNCO

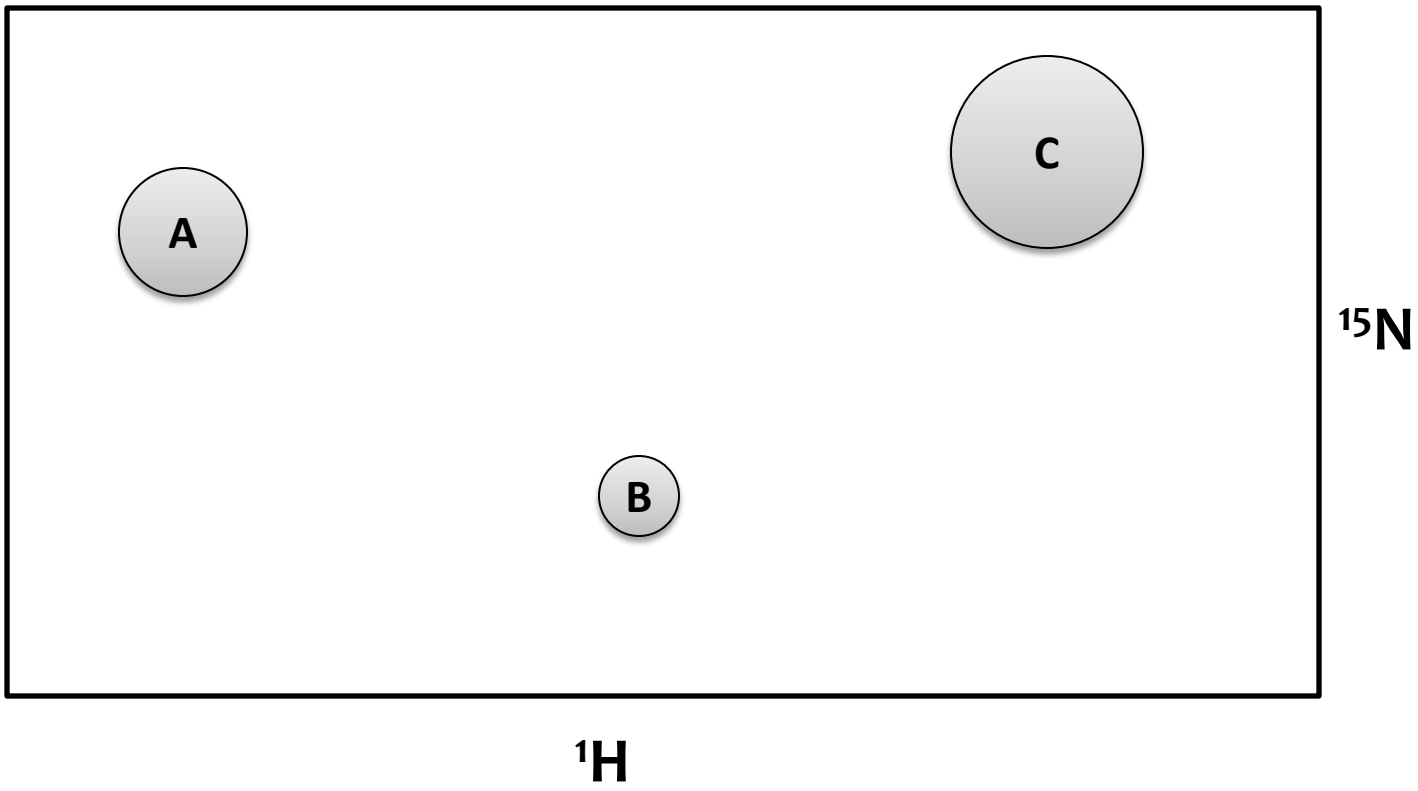
HNCACB / HN(CO)CACB

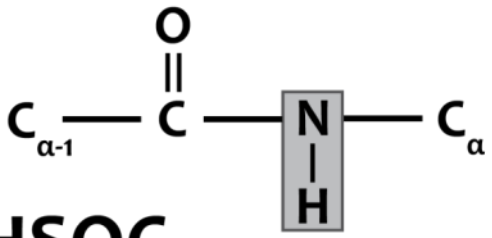


Topics for today:

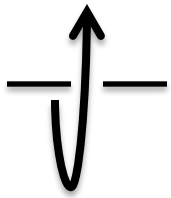
- Sequential walking
- Mapping spin systems to the protein sequence
- Choosing the right triple resonance experiment
- Tricks for resolving challenging systems
  (What to do when the CBCACONH doesn't cut it!)
- Translating assignments to a protein complex
- Software packages for spectra visualization
- Basic primer of the hands-on tutorial
- Using the software: the assignment process demo

Protein Backbone Assignment

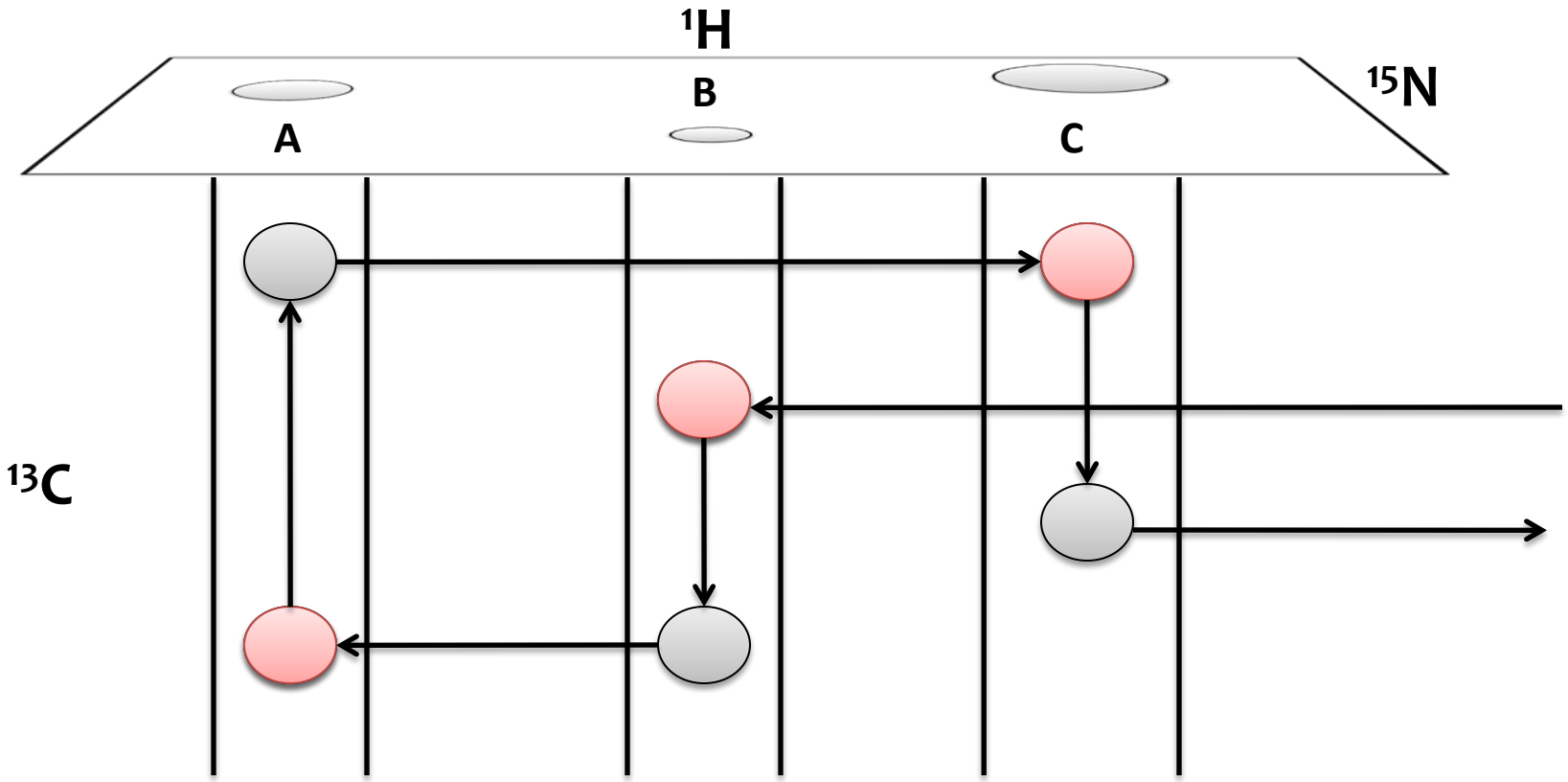




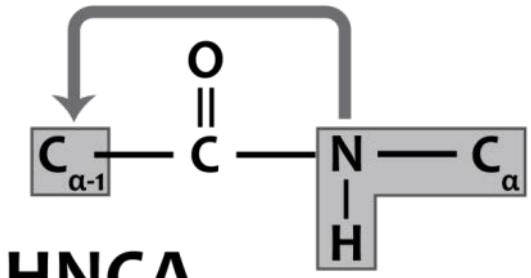
HSQC



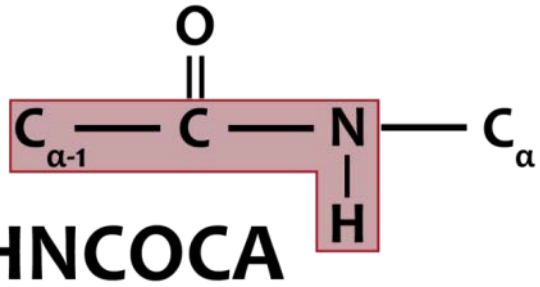
Connecting Spin Systems



B→A→C

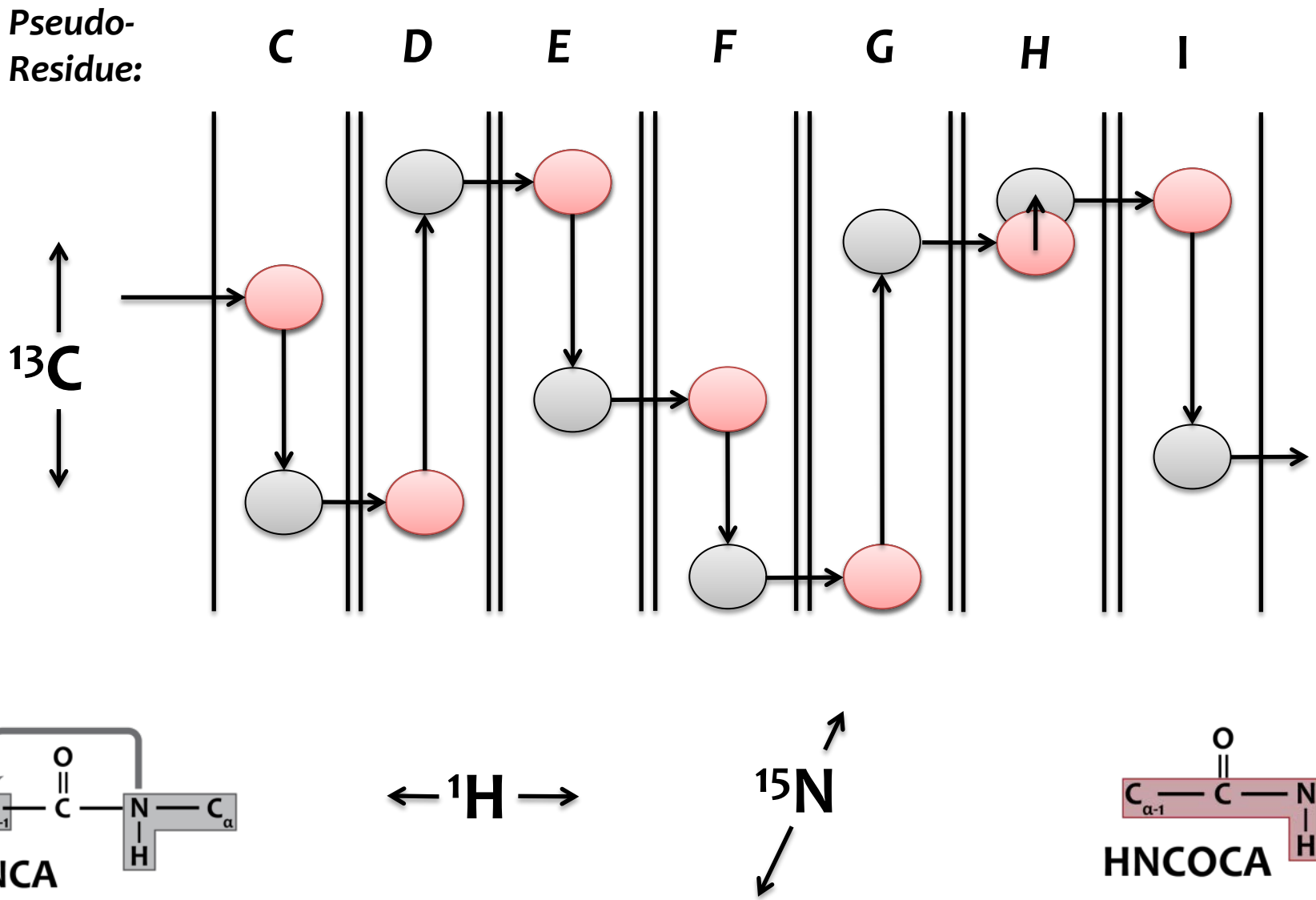


HNCA



HNCOCA

Strip Matching View



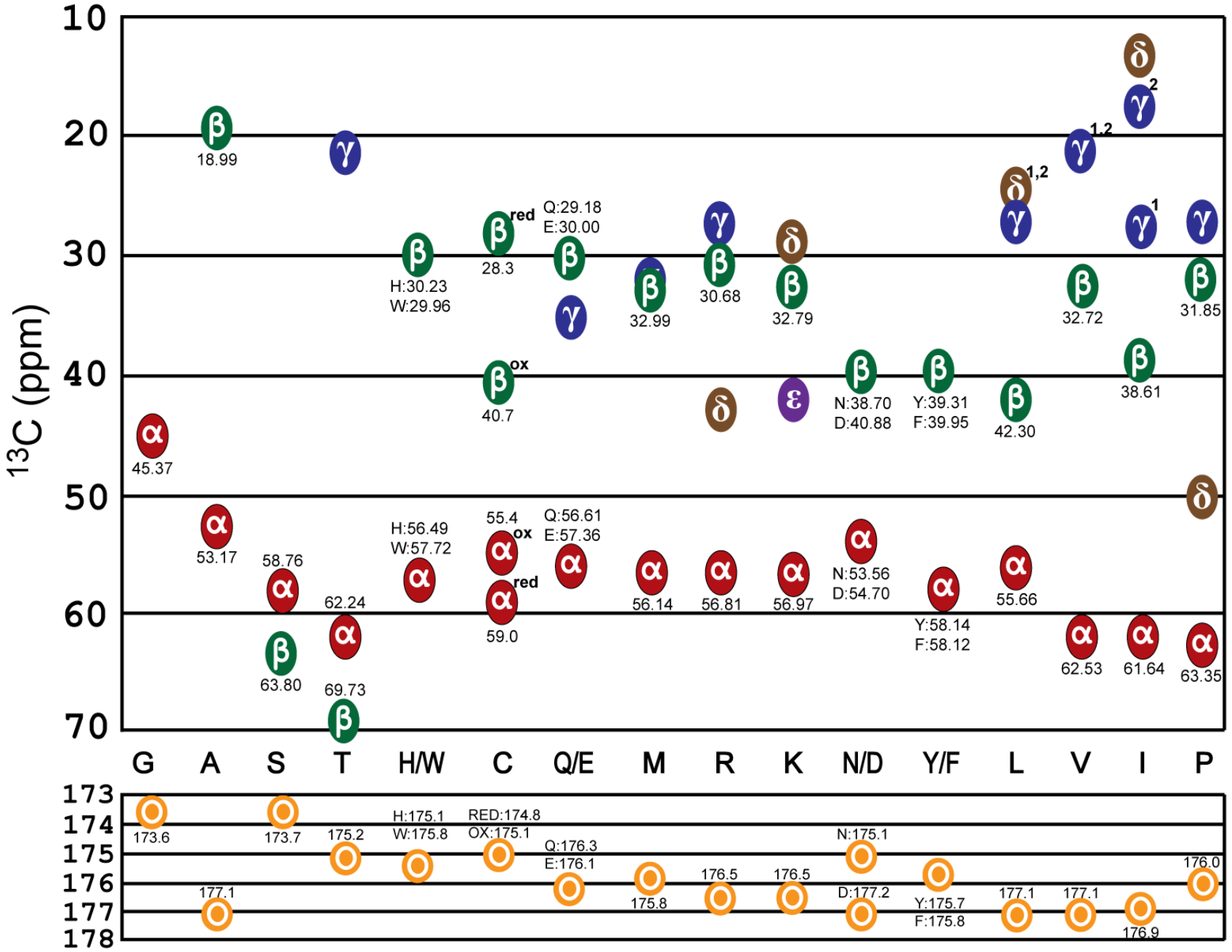
Turning Pseudo-residues to Assignments

Problem:

Need a to convert strips to sequence

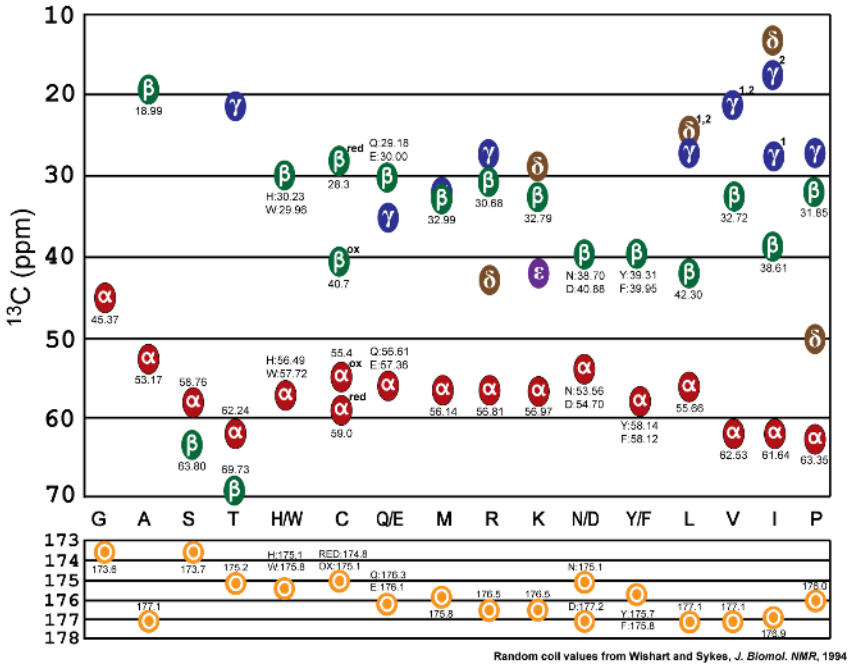
Solution:

1. Determine amino acid identity
2. Compare to known protein sequence



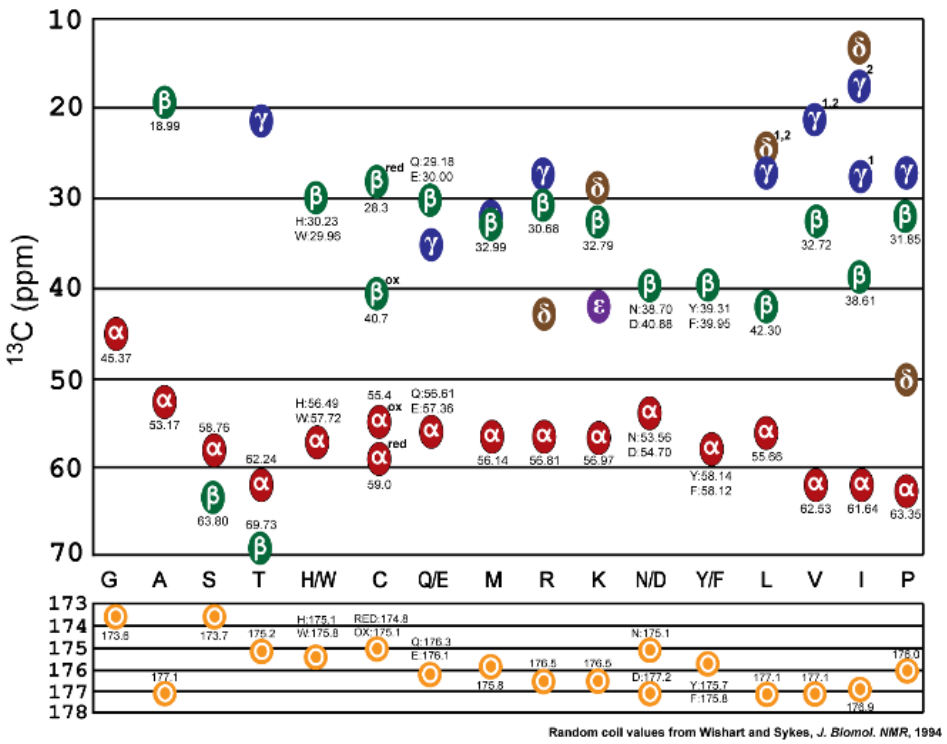
Random coil values from Wishart and Sykes, *J. Biomol. NMR*, 1994

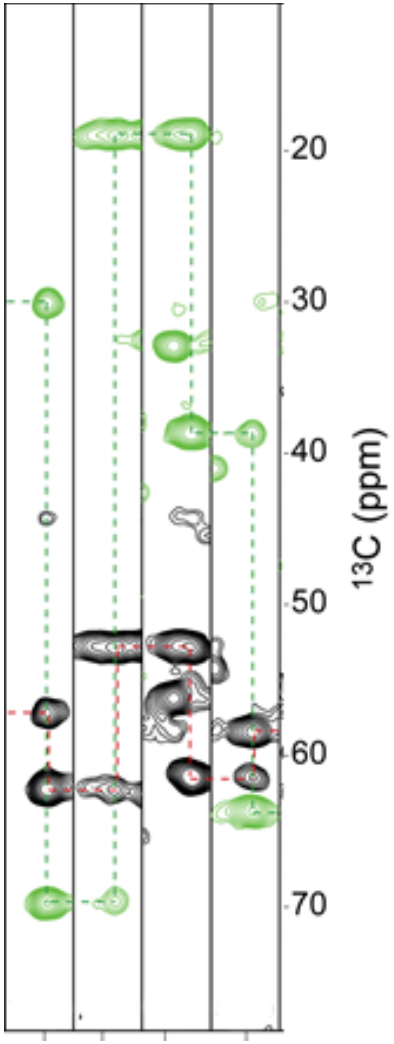
Unique spins of some residue types



- Alanine
  - High alpha (52)
  - Unique beta (19)
- Glycine
  - Unique alpha (45)
  - No beta
- Proline
  - No direct signal (no HN)
  - Can still observe *(i-1)* signal
- Serine/Threonine
  - Unique betas (63/69)
- Asparagine/Aspartate
  - High alpha & Low beta
- Isoleucine
  - Low alpha and low beta
- Valine
  - Low alpha and high beta

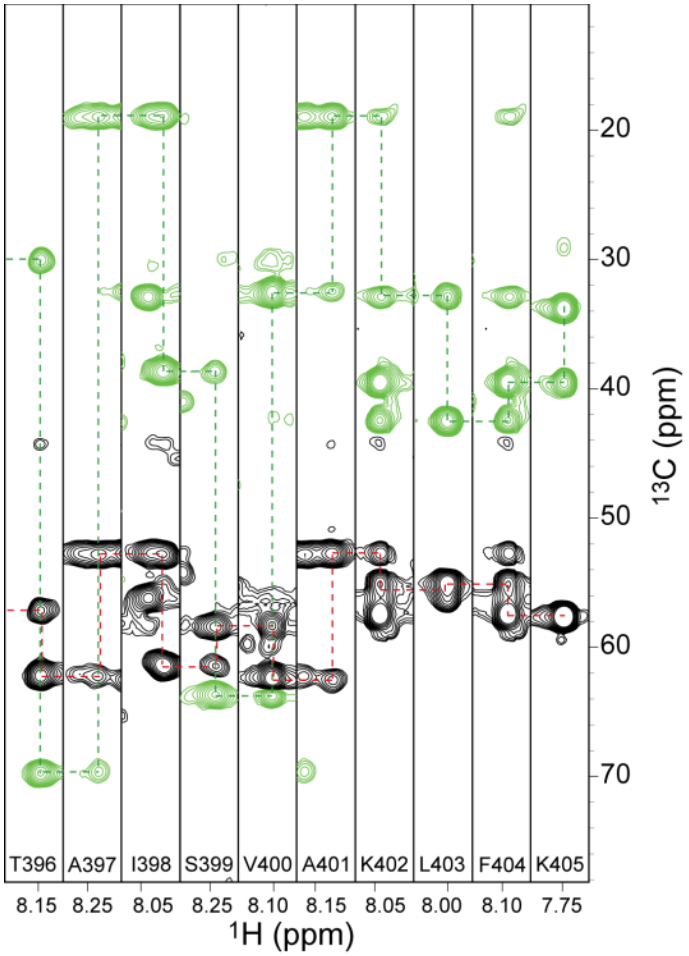
Example Assignment





RERRIHVTQEDFELAVGKVMNKNQETAISVAKLFK

All Spins Need to "Walk"

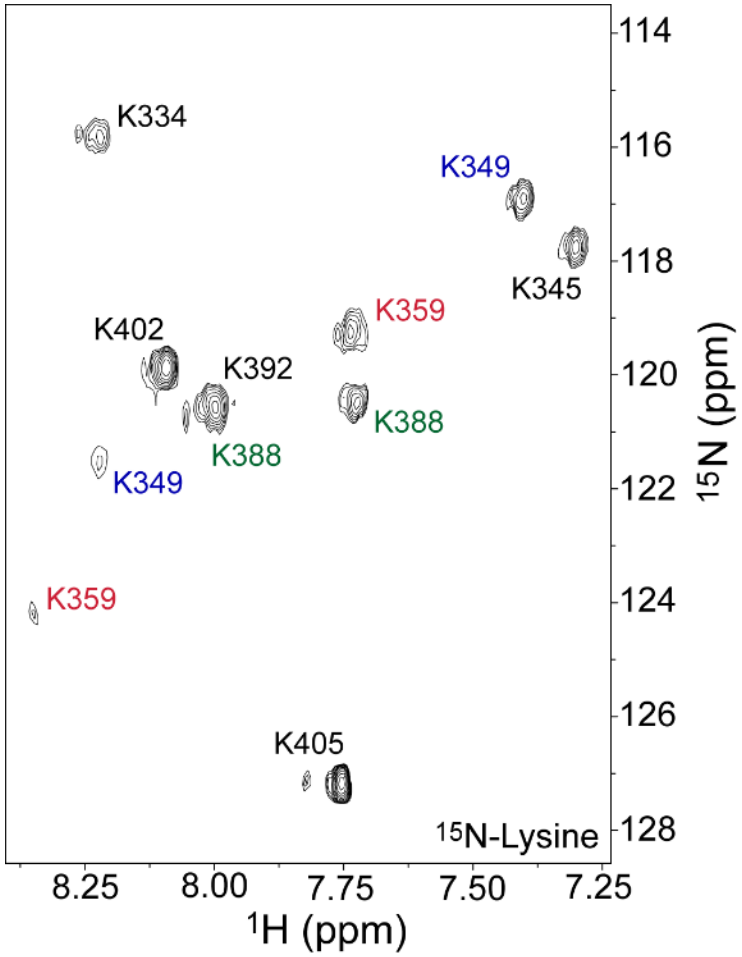


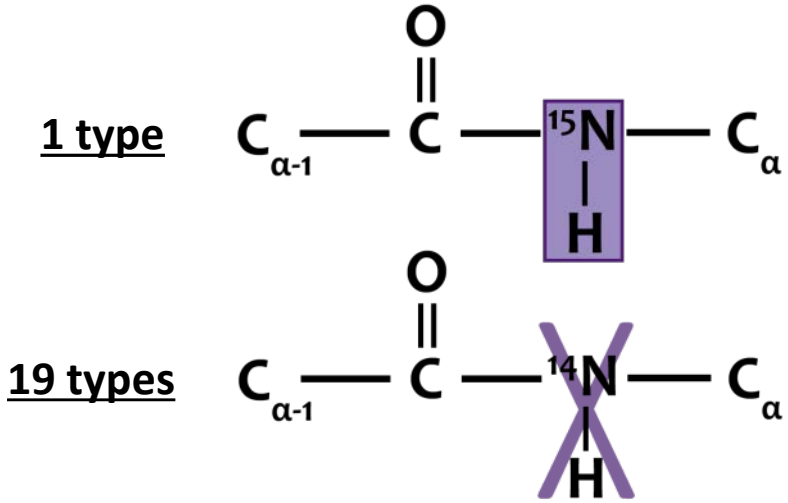
Cross-validate assignments – all spin types will walk if assignments are correct!

Redundant data is safer!

Techniques to interpret tricky assignments

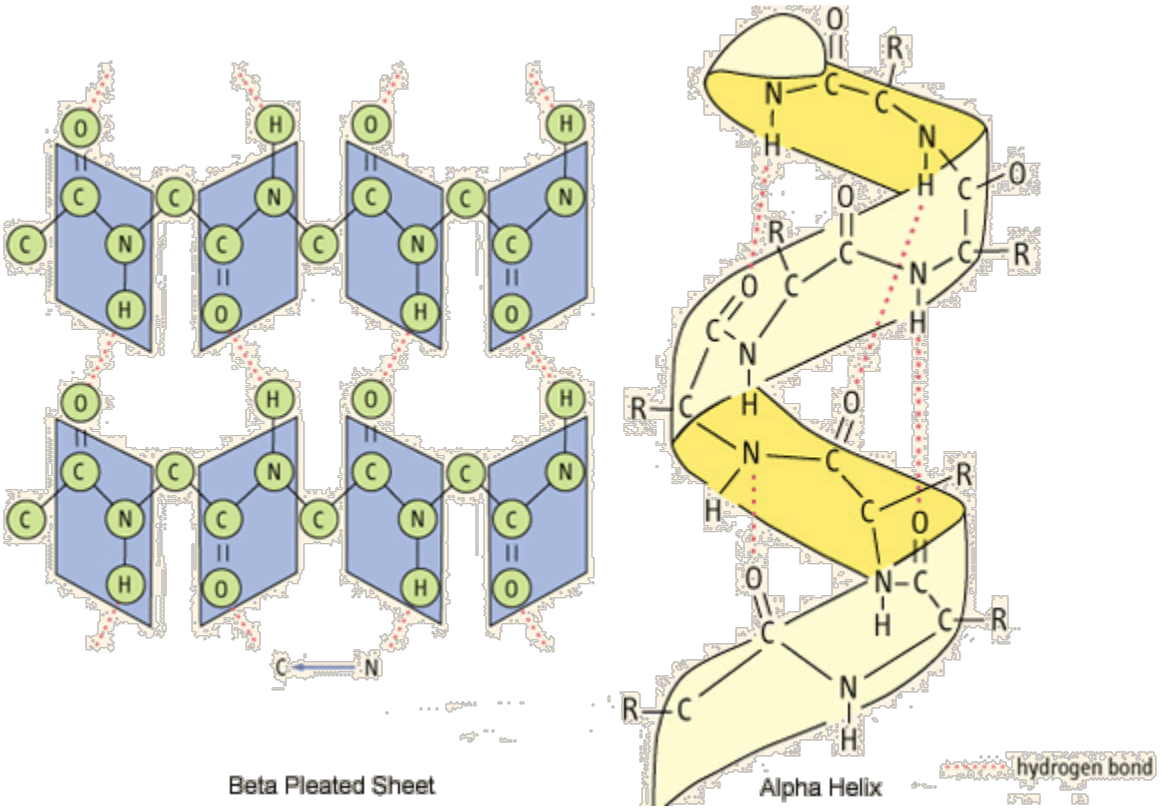
Selective Amino Acid Labeling



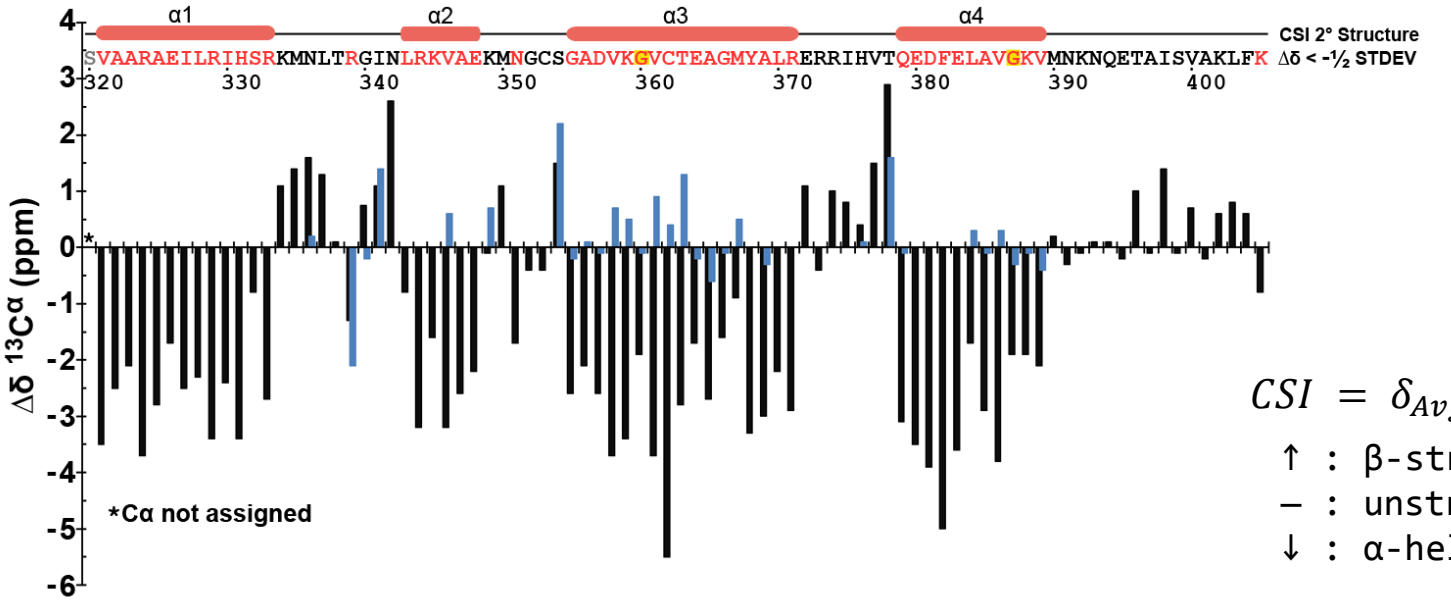


1. Create selectively labeled sample
2. Acquire HSQC/TROSY
3. Identify spin systems of labeled type

Secondary Structure Influences Chemical Environment



Secondary Structure Influences Carbon Chemical Shifts



$$CSI = \delta_{Avg.} - \delta_{Obs.}$$

↑ : β-strand
– : unstructured
↓ : α-helix

If secondary structure is known (i.e. homology model or structure), one can anticipate shifting from the literature random coil value.

Moving back to Homonuclear

^{15}N-dispersed 3D homonuclear expts – relates side chains to amides

- ^{15}N-NOESY has signals unique to 2° - useful for linking pseudo-residues in a helix
  - i.e. α-helicies have i to i+3/i+4 NOE's

- ^{15}N-TOCSY or NOESY shows number of hydrogens in a side chain – useful for amino acid type identity

Practical Considerations

The best way to get good assignments it to collect good data!

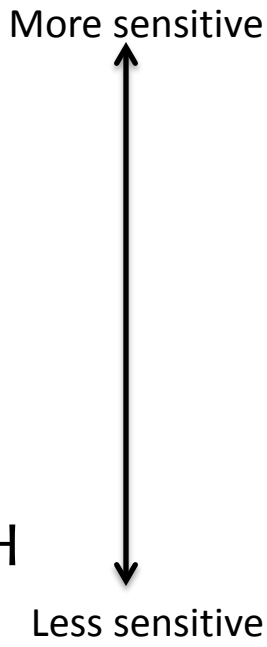
Sample Preparation

- Target 400-600 μM concentration
  - 2x conc = 4x sensitivity
- ^{15}N-^{13}C labeled (~90% ^{2}H preferred)
  - Deuterium slows relaxation, more signal
- Stable for ~4-6 days in acquisition buffer
  - Minimal precipitation/aggregation
  - Sometimes concentration dependent
- Hydrated in water-based buffers (not D_2O or organics) to have signal.
- Salt below 100 mM to take advantage of cryoprobe sensitivity

Spectra Sensitivity

Approximate relative sensitivity:

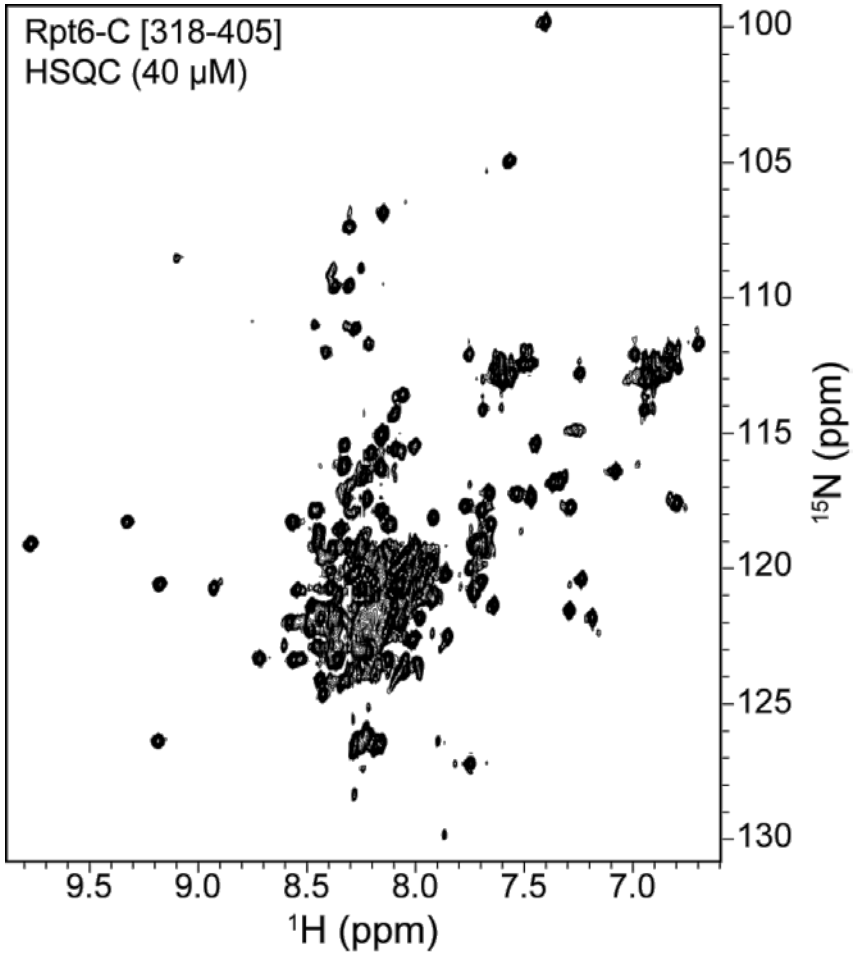
1. HNCO
2. HNCOCA
3. HNCA
4. HNCACB
5. HNCACO
6. CBCACONH



- Non-Uniform sampling (acquire ~25% of points)
- CACB pair gives most data/connectivities, but is least sensitive.
- CA pair is generally more useful than CO pair for connectivities

Spectral Resolution

- HNCO good for determining if H-N peaks are overlapped.
- HNCA has better resolution than HNCACB, good dispersion and smaller frequency range

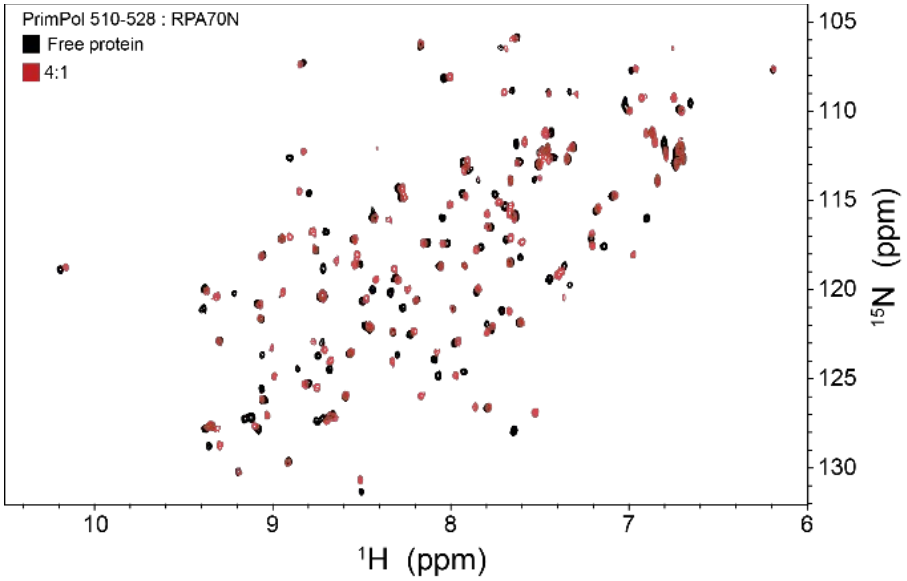


Choosing what spectra to acquire

- Know what assignments you need:
  - Assign HSQC, full backbone, or complete assignment?
- Always start and end acquisition with a ^{15}N-HSQC
  - Checks for protein folding/degradation & sensitivity changes
- H-C plane projections (set ^{15}N resolution to 1)
  - Quick way to see if there is good enough sensitivity before 3D
  - Always test CACB sensitivity first – usually preferred if good sample

Assignment of a Protein Complex

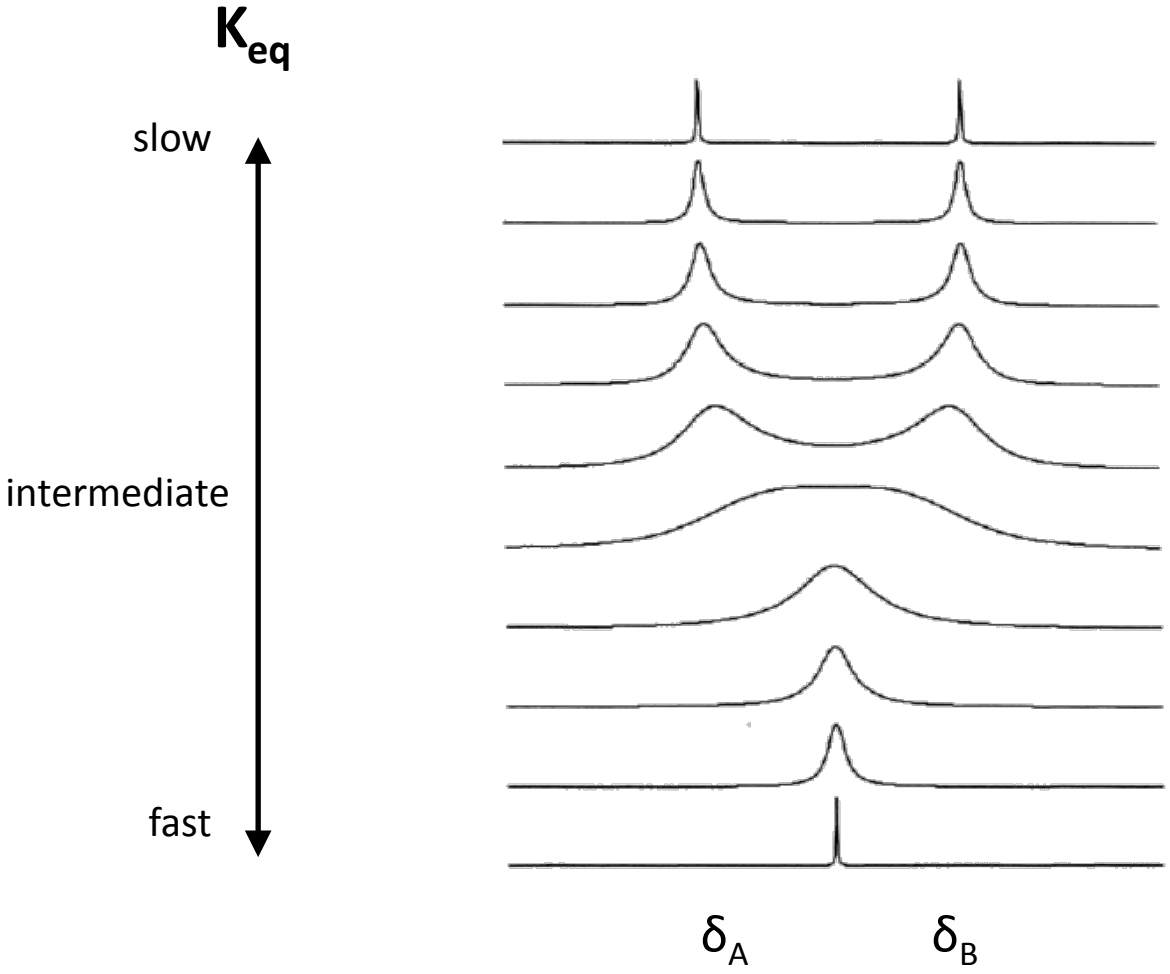
Assignments of a Protein-ligand Complex



Sometimes free and bound spectra are significantly different:

1. Re-assign backbone with triple resonance
2. Translate spin system assignments from free to bound state using amides as a proxy

Interactions: Equilibrium time scale



[A] ≈ [B]

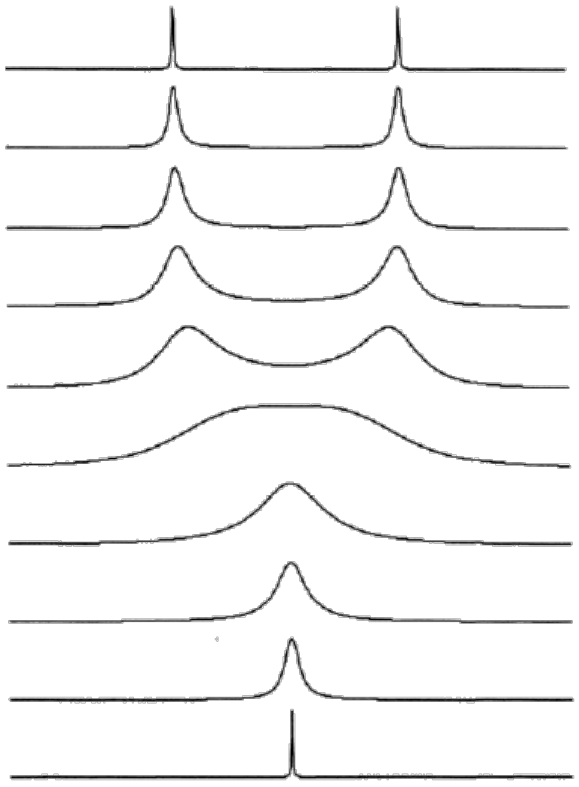
Interactions: Fast Exchange

K_{eq}

slow

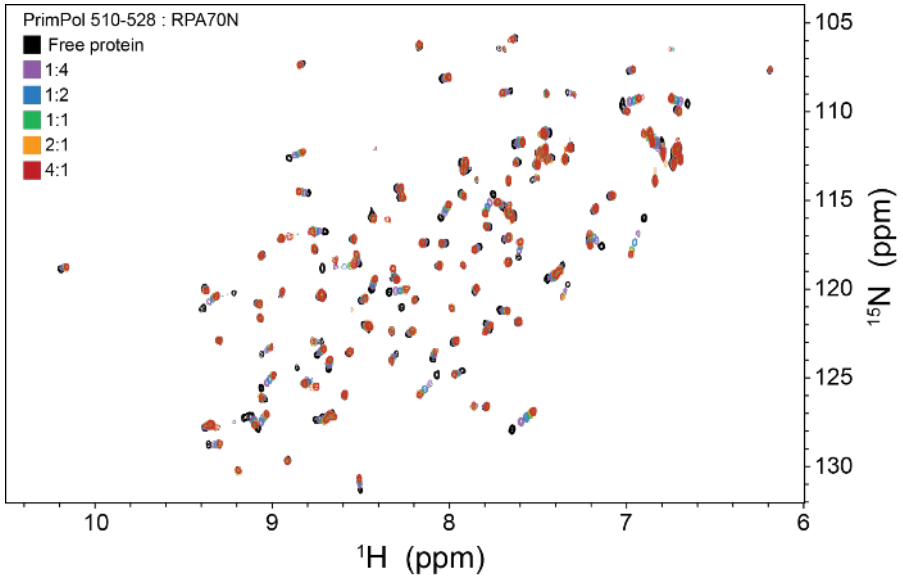
intermediate

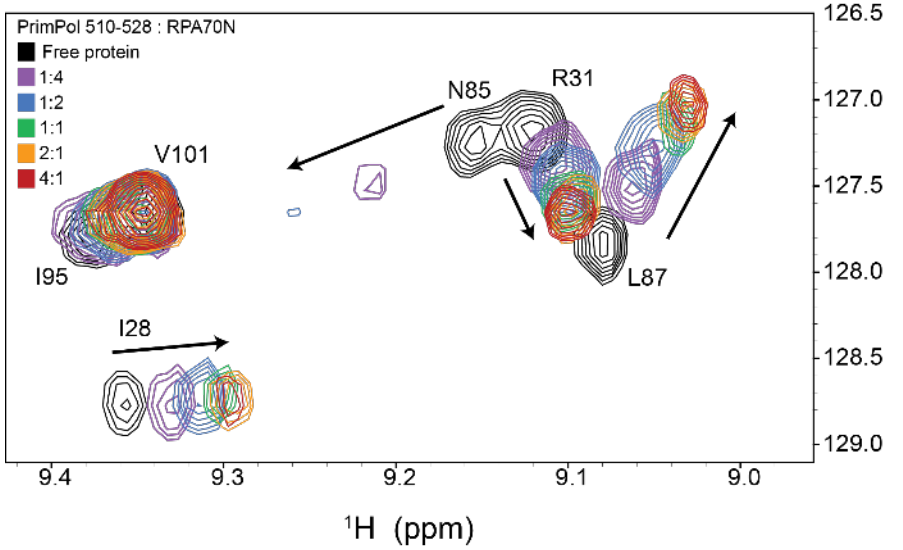
fast



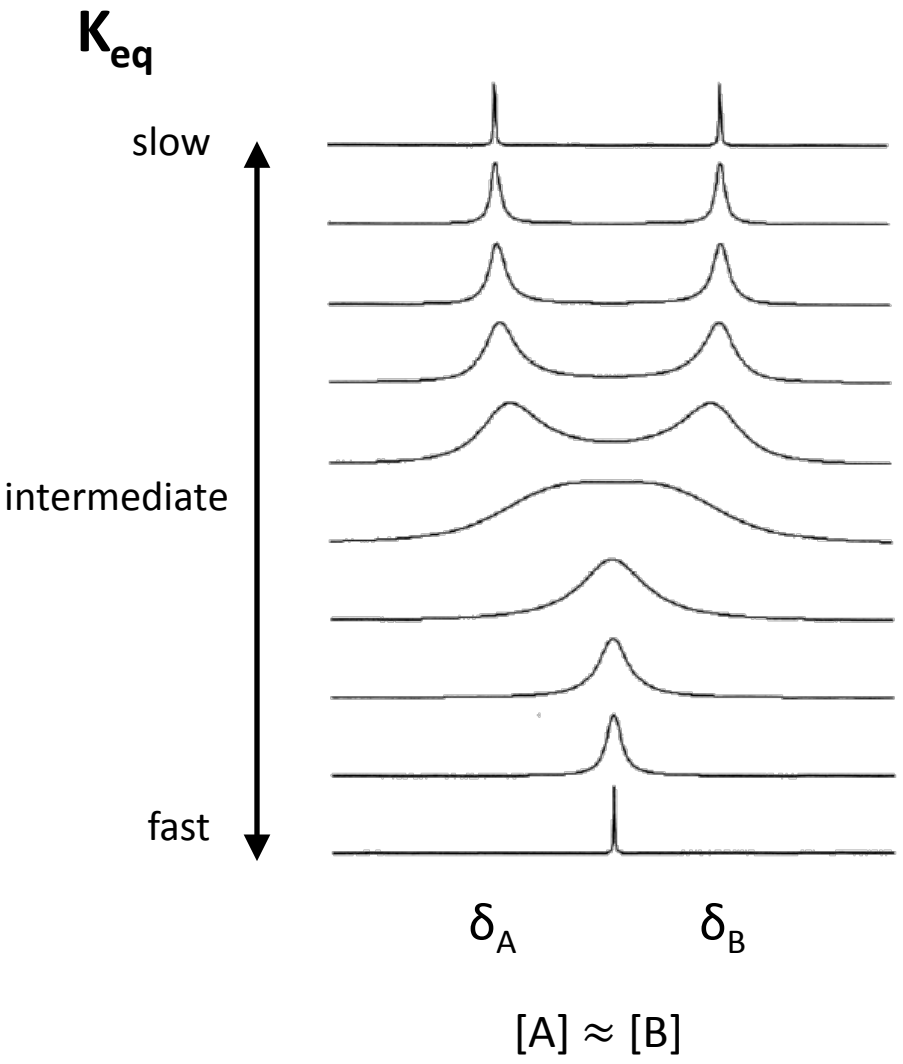
δ_A δ_B

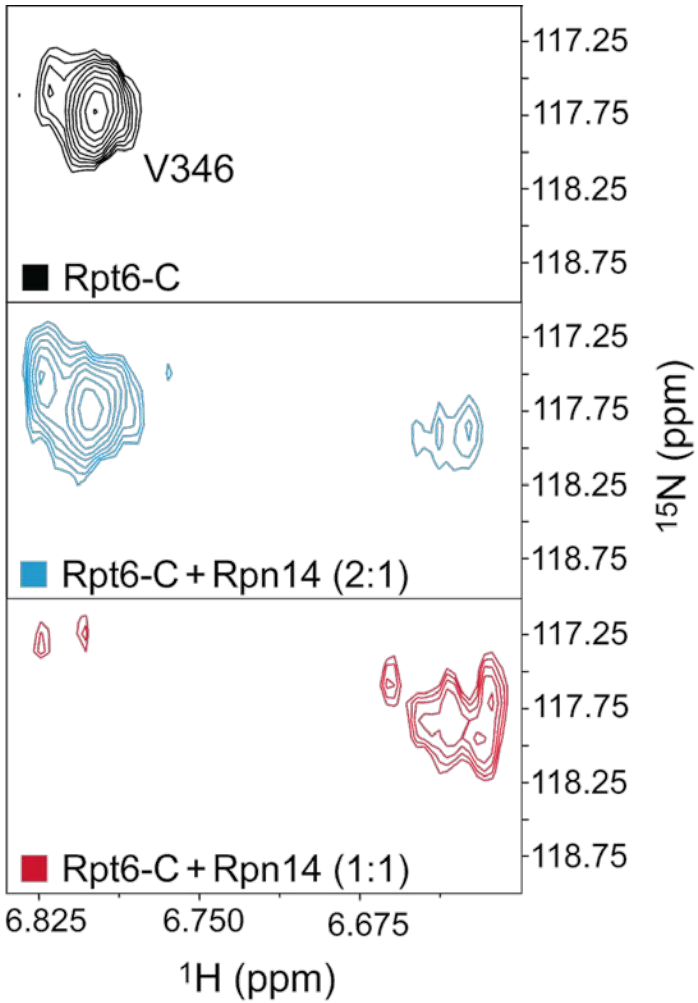
[A] ≈ [B]



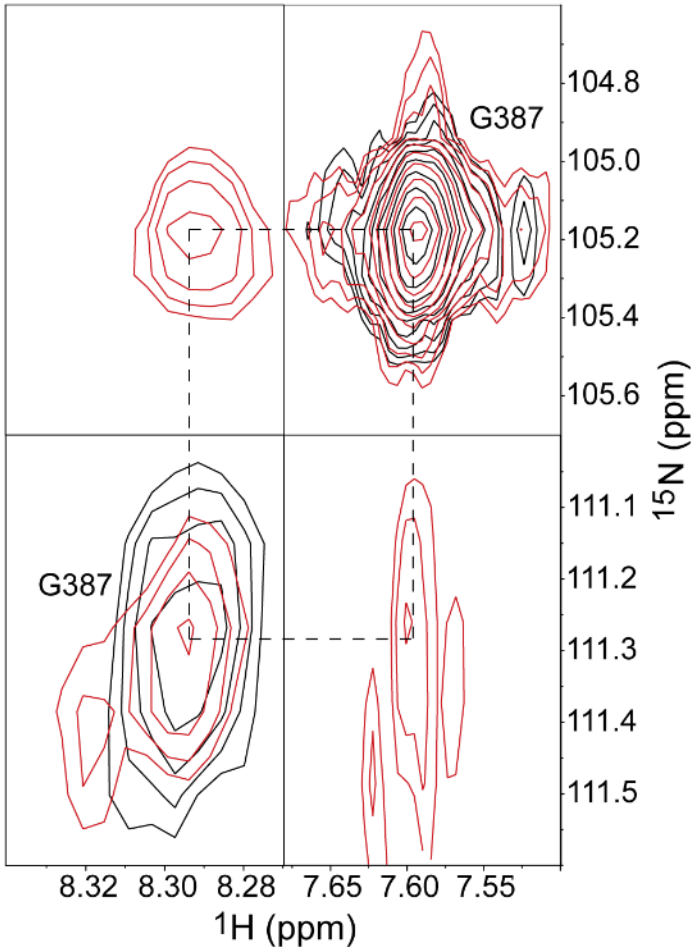


Interactions: Slow Exchange





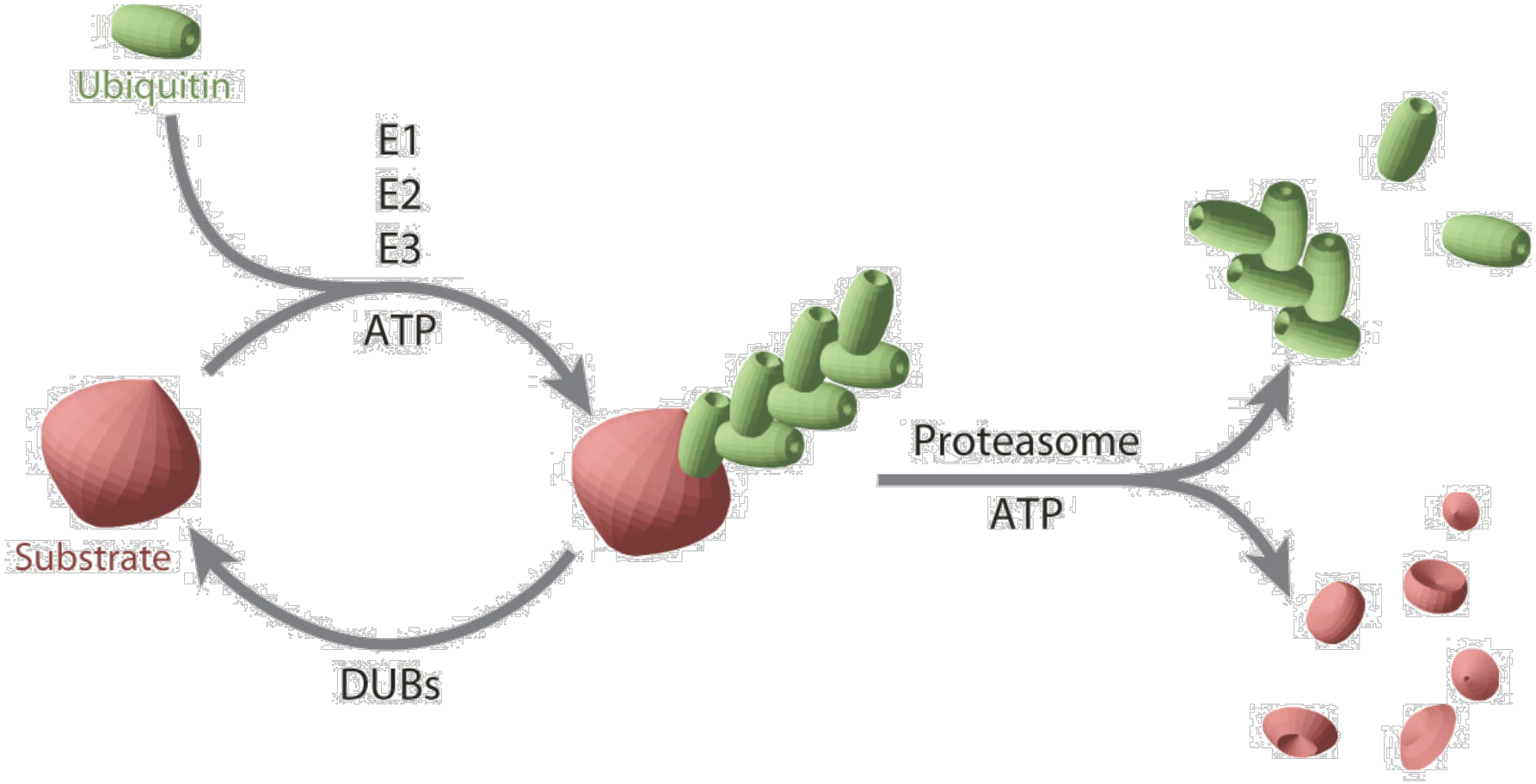
ZZ-Exchange correlations



- Tight interactions (nM dissociation)
- Subsaturating ligand creates two states in slow exchange
- Translate assignment from free state to bound state by identifying cross peaks

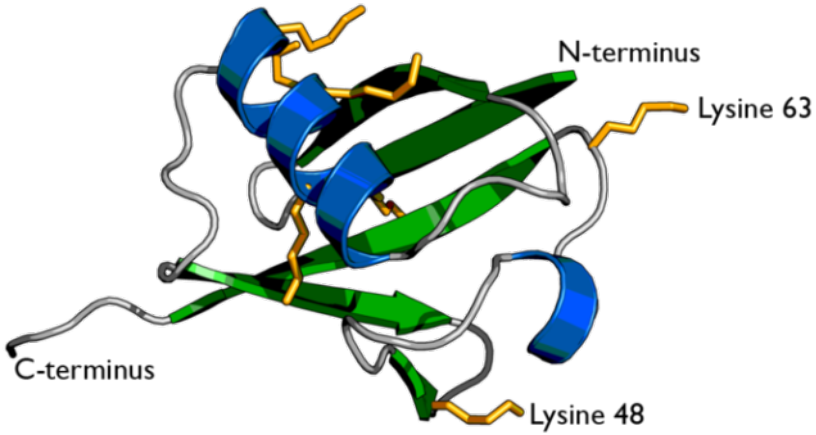
Intro to the Assignment Tutorial

The Ubiquitin Proteasome System



Finley D. (2009) Annu. Rev. Biochem.

Introduction to Ubiquitin



MQIFVKTLTGKTITLEVEPS
DTIENVKAKIQDKEGIPPD
QQRLIFAGKQLEDGRTLSD
YNIQKESTLHLVLRLRGG

- 76 amino acids
- ~8.5 kDa
- Strong/dispersed signal

What are we doing next class?

- Pair into groups of two – meet here to start class
- Find a workstation
- Assign the backbone of ubiquitin
  - 15N-HSQC
  - CBCACONH
  - HNCACB
- Learn visualization software

Visualization Software

- Sparky
- NMR ViewJ
- XEASY
- CARA
- CCPNMR

The Complete Assignment Process

1. View HSQC
2. Generate (pick) peaks on HN correlations
3. Look into carbon plane for each given H and N ppm
4. Pick peaks for each carbon resonance
5. Use overlays to determine i and i-1
6. Sequential walking
7. Amino acid type identification
8. Map strip series to sequence
9. Verify assignments with every acquired spectrum
10. Determine side chain assignments, if necessary.

Demonstration