Preview of Practical Solution NMR, Up Through 2-D HSQC AND TROSY, Including a TROSY Gallery

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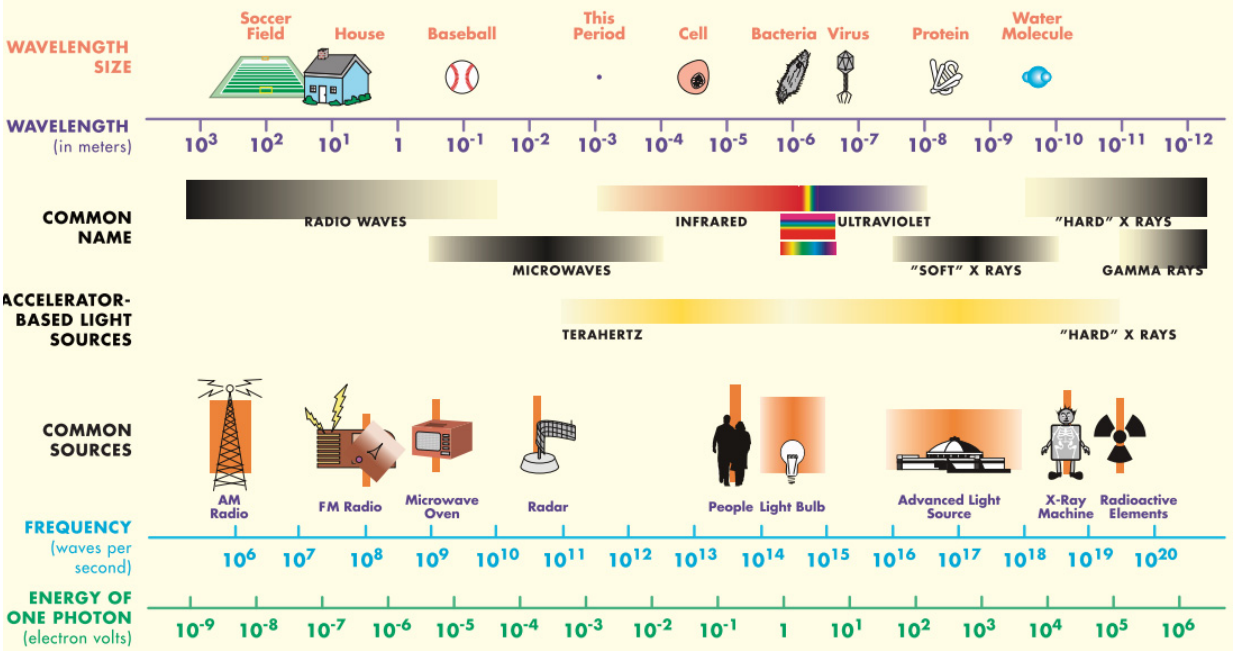
(With Many Thanks to Dr. Markus Voehler and Catherine Deatherage of Vanderbilt for Significant Edits)

This is an elementary introduction to NMR that is designed to help complete newcomers to NMR to grasp just enough about the main physical and experimental features of NMR to facilitate their early experience in collecting and interpreting simple 1- and 2-D spectra (especially 2-D $^{1}H,^{15}N$-HSQC AND TROSY). Some of the approximations used in describing NMR theory here are grossly oversimplified and in some cases not even completely accurate (deplorable!). The theory presented herein is intended to be only a practical intellectual "placeholder" to help one prepare for first NMR experiments, a placeholder that should be replaced by a deeper and more accurate understating of how NMR and its related experiments work. After digesting this document, I recommend sections of the following books for further reading. (There are also excellent books on advanced NMR not listed here).

Modern NMR Spectroscopy, A Guide for Chemists (Jeremy Sanders and Brian Hunter, 1993)
Pulse and Fourier Transform NMR (Thomas Farrar and Edwin D. Becker, 1971; still relevant)
Applied NMR Spectroscopy for Chemists and Life Scientist (Oliver Zerbe and Simon Jurt, 2014)
Nuclear Magnetic Resonance Spectroscopy (Frank Bovey, 1988)
Biomolecular NMR Spectroscopy (Jeremy Evans, 1995)
Understanding NMR Spectroscopy (James Keeler, 2010)
NMR of Proteins and Nucleic Acids (Kurt Wuthrich, 1986)
Fundamentals of Protein NMR Spectroscopy (Gordon Rule and T. Kevin Hitchens, 2006)

Matter will absorb electromagnetic radiation ("light") over an astounding range of frequencies and wavelengths. Nuclear magnetic resonance is based on the fact that the nuclei of certain atom types will absorb radiation in the radiofrequency range: light frequencies on the order of 100-600 megahertz (mega = 10^6, Hz = waves per sec^{-1}) which means wavelengths of roughly 200-5000 cm (λ=c/ν), pretty long—you can't see this "light", but it can be detected with a radio.).

Figure: From Advanced Light Source http://www-als.lbl.gov/images/stories/About_the_ALS/quick-facts-page.pdf



This range of frequencies provides a clue regarding one of the reasons NMR is so useful in the study of biological systems: the frequency ranges dealt with in NMR are on the same order of magnitude as the frequencies (rates) of many biological processes. For reasons we haven't time to delve into, this endows NMR spectroscopy with unique abilities to observe both *static* and *dynamic* biological structures and processes. NMR can yield information on a wide range of interesting topics:

- Chemical Structure (what is the chemical identity of an unknown sample?)
- Quantity: What are the relative concentrations of molecules in a mixture?
- 3-D Structure: What is the conformation of a molecule?
- Dynamic Structure: What motions is the molecule undergoing?
- Kinetics: What is the rate of a process?
- Mapping: Where is the binding site in a protein? What is the topology of a membrane protein?
- "Reporting": NMR signals can be employed as tools for determining binding constants, monitoring protein denaturation, monitoring phase transitions, etc.

Note that both solids and liquids can be observed with NMR, although they give rise to characteristically different classes of spectra. Here we will focus on "solution" liquid state NMR, not *solid state* NMR.

Note also that NMR is non-invasive, meaning it does not perturb the system under observation (unlike some fluorescence, EPR, and other experiments that require the introduction of an unnatural probe). NMR can therefore be used in studies of both *in vivo* and *in vitro* systems.

Some Basic NMR Theory

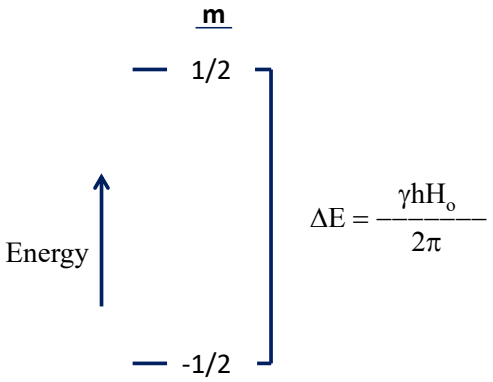
NMR active nuclei have a specific property, referred to as "spin" because NMR is fundamentally related to the "spin" state of the nucleus (where spin refers to the precession of the nuclear magnetic moment about the magnetic field director). Isotopes that have "lopsided" nuclei have nuclear spin quantum numbers (I) that are non-zero (usually I=1/2 for biologically relevant nuclei). In a magnetic field the possible energy associated with the spinning of a "lop-sided" nucleus will be "quantized" into 2I+1 states. For I=1/2 nuclei such as ^{1}H, ^{31}P, ^{15}N, and ^{13}C, 2I+1 means the energy will be either of 2 states:

The energy between the two possible states is given as:

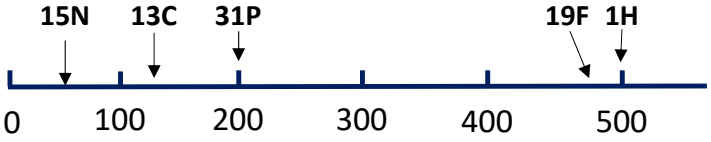
$$\Delta E = h\nu = \gamma h H_o/2\pi$$

where:

- h is Planck's constant (look it up in a physics book)
- H_o is the magnetic field strength (usually in Tesla)
- γ is known as the magnetogyric ratio and is nucleus-specific (each type of nucleus has its own value)
- ν is known as the "Larmor frequency" and is equal to γH_o



For a NMR magnet of 11.7 Tesla the Larmor resonance frequencies of some relevant nuclei are listed:



Colloquially, an 11.7 Tesla magnet is known as a "500 MHz" magnet because this is the ^{1}H resonance frequency of this magnet. Modern NMR magnets are usually cryogenically cooled superconducting magnets

spanning 2-23.4 Tesla (a "1000 MHz instrument" is currently the largest available NMR magnet- 23.4T).

In a magnetic field, the spins align along the field either parallel or anti-parallel (-1/2 or +1/2). The bulk of the spins are in equilibrium between these states, with a net only a few more spins populating the energetically favored -1/2 state. This explains why NMR is a method of relatively low sensitivity, one of its biggest obstacles. "Resonance" occurs when a nucleus in the m= -1/2 spin quantum state absorbs RF energy of energy = ΔE (= $h\nu$) so that the nucleus transitions to the higher energy m=1/2 state. The NMR detector can sense this absorption of energy along with its characteristic frequency. A computer converts the signal received by the detector into data, which you can look either as "raw data" intensity versus time decaying waveforms, or after Fourier Transformation as frequency dependent intensity spectra. Thus, the nucleus gives off a characteristic "signal" or "peak" in response to its RF energy absorption at its resonant frequency.

One might wonder if NMR is limited because this simple theory predicts that *all* nuclei of a certain type (such as all protons) within a sample will give rise to a signal at the exact same frequency, ν. Fortunately, this is not the case! It turns out that each nucleus doesn't experience a magnetic field of *exactly* H_o. Instead, the *local electronic (chemical) environment* of the nucleus attenuates the magnetic field experienced by the nucleus by a factor of $(1-\sigma)$, where σ is sometimes referred to as the "shielding constant". As a result the actual resonance frequency becomes equal to:

$$\nu = \frac{\gamma \cdot H_o \cdot (1 - \sigma)}{2\pi}$$

(Remember, 1 Hz = 1 per second = 1 sec^{-}). σ is a measure of the shift in magnetic field strength experienced by a nucleus due to its local chemical environment (the "absolute chemical shift", slightly different from the observed chemical shift—defined below—which is what we work with on a daily basis.

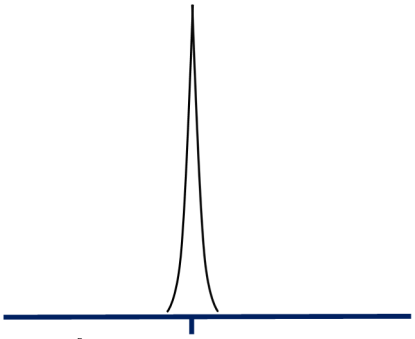
Why is a Large NMR Magnet Generally Preferred?

Anyone who has conducted an NMR experiment is familiar with chemical shift units. Usually, NMR people don't plot spectra as NMR *frequency* vs. signal intensity. Instead, data is plotted in terms of observed chemical shift σ_{obs} (in ppm) vs. signal intensity, where σ_{obs} being defined as:

$$\sigma_{obs} = \frac{\nu - \nu_{ref}}{\nu_{ref}} \; x\, 10^6 \;, \quad \text{which can be rearranged:} \quad \sigma_{obs}/10^6 = \frac{\nu - \nu_{ref}}{\nu_{ref}}$$

Here ν is the absolute resonance frequency and ν_{ref} is the absolute resonance frequency of a reference (which by definition corresponds to σ_{obs} = 0 PPM. The numerator (ν - ν_{ref}) is on the order of 10^6 times smaller than ν_{ref}, hence the introduction of the factor $1/10^6$, which is why chemical shifts are expressed in dimensionless units of part per million or PPM (for example water has a 1H chemical of ca. 4.7 PPM).

Why use PPM instead of frequency units (Hz)? Because PPM for a given species will always be the same, regardless of H_o. If absolute frequency is used, the absolute frequency for a given species will be dependent upon the strength of the magnet used in the NMR experiment. Also, PPM tend to be nice little numbers instead of the large numbers associated with absolute frequency. This is illustrated:



If H_o = 11.7T → 1.7 PPM, ν = 500,130,450 Hz, σ_{obs} = 850.2 Hz
If H_o = 5.87T → 1.7 PPM, ν = 250,965,225 Hz, σ_{obs} = 426.6 Hz

The fact that the absolute resonance frequency is H_o-dependent leads into a consideration of magnetic field strength. It turns out that a *larger* (and more expensive!) magnet is generally preferred in NMR spectroscopy for several reasons. First, larger magnets give better sensitivity, meaning quality spectra can be acquired in shorter time periods because:

NMR Resonance Signal-to-Noise Ratio (S/N) is proportional to $\gamma^{5/2} \cdot H_o^{3/2}$

(from this expression we also see that high γ nuclei like protons are inherently much more sensitive than low γ nuclei like ^{15}N.)

Table 1.2 Magnetic properties of some biologically useful nuclei

Isotope	Spin	Natural abundance (%)	Quadrupole moment Q (10^{-28} m^2)	Gyromagnetic ratio γ (10^7 rad s^{-1} T^{-1})	Sensitivity rel.[a]	abs.[b]	NMR-frequency (MHz) at a field (T) of 2.3488
^{1}H	1/2	99.98	—	26.7522	1.00	1.00	100.000
^{2}H	1	1.5×10^{-2}	2.87×10^{-3}	4.1066	9.65×10^{-3}	1.45×10^{-6}	15.351
^{3}H	1/2	0	—	28.5350	1.21	0	106.663
^{7}Li	3/2	92.58	-3.7×10^{-2}	10.3976	0.29	0.27	38.863
^{11}B	3/2	80.42	4.1×10^{-2}	8.5847	0.17	0.13	32.084
^{13}C	1/2	1.108	—	6.7283	1.59×10^{-2}	1.76×10^{-4}	25.144
^{14}N	1	99.63	1.67×10^{-2}	1.9338	1.01×10^{-3}	1.01×10^{-3}	7.224
^{15}N	1/2	0.37	—	−2.7126	1.04×10^{-3}	3.85×10^{-6}	10.133
^{17}O	5/2	3.7×10^{-2}	-2.6×10^{-2}	−3.6280	2.91×10^{-2}	1.08×10^{-5}	13.557
^{19}F	1/2	100	—	25.1815	0.83	0.83	94.077
^{23}Na	3/2	100	0.10	7.0704	9.25×10^{-2}	9.25×10^{-2}	26.451
^{25}Mg	5/2	10.13	0.22	−1.6389	2.67×10^{-3}	2.71×10^{-4}	6.1195
^{31}P	1/2	100	—	10.8394	6.63×10^{-2}	6.62×10^{-2}	40.481
^{35}Cl	3/2	75.53	-8.2×10^{-2}	2.6242	4.70×10^{-3}	3.55×10^{-3}	9.798
^{39}K	3/2	93.1	5.5×10^{-2}	1.2499	5.08×10^{-4}	4.73×10^{-4}	4.667
^{43}Ca	7/2	0.145	-5×10^{-2}	−1.8028	6.40×10^{-3}	9.28×10^{-6}	6.728
^{51}V	7/2	99.76	2.17×10^{3}	-5.2×10^{-2}	0.38	0.38	26.289
^{57}Fe	1/2	2.19	—	0.8687	3.37×10^{-5}	7.38×10^{-7}	3.231
^{75}As	3/2	100	0.29[c]	4.5961	2.51×10^{-2}	2.51×10^{-2}	17.126
^{77}Se	1/2	7.58	—	5.1214	6.93×10^{-3}	5.25×10^{-4}	19.067
^{113}Cd	1/2	12.26	—	−5.9609	1.09×10^{-3}	1.33×10^{-3}	22.182

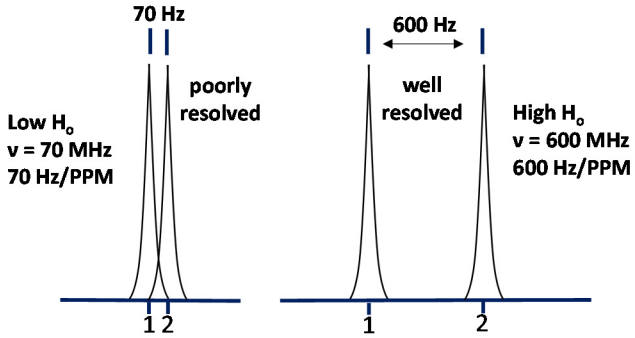
[a] At constant field for equal number of nuclei.
[b] Product of relative sensitivity and natural abundance.

Table from book by Jeremy Evans

A second reason for preferring as large an H_o as possible is the spectral resolution. Remember...

- PPM is the same regardless of H_o
- Hz/PPM varies linearly with H_o
- NMR peak line widths usually undergo only modest changes with varying H_o

Let us consider a hypothetical case of two resonances, which have line widths of 50 Hz and are separated by 1 PPM:



We see that even though the two peaks have the same linewidth at both fields (in this example) that they are not well resolved in the spectrum from a low magnetic field spectrometer, but are well resolved with a high field magnet is used.

A third reason higher field magnets are preferred is that the TROSY effect becomes active only at very high fields. As we will discuss later, the TROSY effect sometimes allows very high quality NMR spectra to be acquired for very large molecules and complexes.

Typical PPM Ranges for Various Nuclei and Chemical Moieties

As we discussed above, the chemical shift displayed by a nucleus is dependent on its chemical environment. Thus, certain chemical moieties are associated with certain chemical shift ranges for given nuclei.

The chemical shift reference compound for proton NMR is tetramethylsilane (0 PPM). This same compound serves as a reference for ^{13}C NMR (0 PPM). For ^{31}P NMR, phosphoric acid is 0 PPM.

In the case of ^{1}H NMR, aromatic groups typically resonate in the 6.5-9 PPM range ("downfield", methyl groups near 1 PPM ("upfield"), etc. The next pages provide a typical range of chemical shifts for several nuclei relevant to protein NMR. Furthermore, the Biological Magnetic Resonance Data Bank (BMRB) provides up-to-date chemical shift statistic and other information for amino acids, RNA, and DNA at http://www.bmrb.wisc.edu/ref_info/

Table 1.3. Nitrogen chemical shifts. The nitrogen chemical shifts for side-chain atoms are shown. The amide nitrogen chemical shifts are ≈ 120 ppm, with the exception of glycine, which is found at 109.9 ppm. Data from BioMagResBank [52].

Residue	*Shifts*	*Residue*	*Shifts*
Arg	89.8 (ϵ), 74.8 NH1, 75.8 NH2	His	190.7 (δ1), 179.8 (ϵ2)
Asn	112.8 (δ)	Lys	71.86 (ζ)
Gln	111.8 (ϵ)	Trp	129.5 (ϵ)

Table 1.4. Carbon chemical shifts. The average carbon chemical shifts were obtained from the BioMagResBank [52]. Carbonyl shifts have been omitted from this table since they are quite uniform at approximately 175 ppm.

Residue	C_α	C_β	*Others*
Gly	45.3		
Ala	53.1	18.9	
Val	62.5	32.6	21.3 (CH3)
Ile	61.6	38.6	27.6 (γ1), 17.3 (γCH3), 13.4 (δCH3)
Leu	55.7	42.3	26.8 (γ), 24.5 (δCH3)
Pro	63.3	31.8	27.1 (γ), 50.3 (δ)
Ser	58.6	63.8	
Thr	62.1	69.6	21.4 (γCH3)
Asp	54.5	40.7	178.41 (γ) sidechain
Glu	57.4	30.0	36.0 (γ), 181.9 (δ) sidechain
Lys	56.8	32.8	24.9 (γ), 28.8 (δ), 40 (ϵ)
Arg	56.9	30.7	27.3 (γ), 43.1 (δ), 159.0 (ζ)
Asn	54.5	40.7	178.41 (γ) sidechain
Gln	56.6	29.1	33.7 (γ), 179.7 (δ) sidechain
Met	56.1	32.9	32.1 (γ), 17.2 (ϵCH3)
Cys	57.4	34.1	
Trp	57.7	30.1	110-137 (aromatic)
Phe	58.2	40.0	129-138 (aromatic)
Tyr	58.0	39.1	117 (ϵC), 132 (δC), 156 (ζ)
His	56.4	30.0	119.8 (δ2), 136 (ϵ1)

From: Rule and Hitchens book

TABLE 2.3. Random Coil ^1H Chemical Shifts for the 20 Common Amino Acid Residues[a]

Residue	NH	αH	βH	Others
Gly	8.39	3.97		
Ala	8.25	4.35	1.39	
Val	8.44	4.18	2.13	γCH_3 0.97, 0.94
Ile	8.19	4.23	1.90	γCH_2 1.48, 1.19
				γCH_3 0.95
				δCH_3 0.89
Leu	8.42	4.38	1.65,1.65	γH 1.64
				δCH_3 0.94, 0.90
Pro[b]		4.44	2.28,2.02	γCH_2 2.03, 2.03
				δCH_2 3.68, 3.65
Ser	8.38	4.50	3.88,3.88	
Thr	8.24	4.35	4.22	γCH_3 1.23
Asp	8.41	4.76	2.84,2.75	
Glu	8.37	4.29	2.09,1.97	γCH_2 2.31, 2.28
Lys	8.41	4.36	1.85,1.76	γCH_2 1.45, 1.45
				δCH_2 1.70, 1.70
				εCH_2 3.02, 3.02
				εNH_3^+ 7.52
Arg	8.27	4.38	1.89,1.79	γCH_2 1.70, 1.70
				δCH_2 3.32, 3.32
				NH 7.17, 6.62
Asn	8.75	4.75	2.83,2.75	γNH_2 7.59, 6.91
Gln	8.41	4.37	2.13,2.01	γCH_2 2.38, 2.38
				δNH_2 6.87, 7.59
Met	8.42	4.52	2.15,2.01	γCH_2 2.64, 2.64
				εCH_3 2.13
Cys	8.31	4.69	3.28,2.96	
Trp	8.09	4.70	3.32,3.19	2H 7.24
				4H 7.65
				5H 7.17
				6H 7.24
				7H 7.50
				NH 10.22
Phe	8.23	4.66	3.22,2.99	2,6H 7.30
				3,5H 7.39
				4H 7.34
Tyr	8.18	4.60	3.13,2.92	2,6H 7.15
				3,5H 6.86
His	8.41	4.63	3.26,3.20	2H 8.12
				4H 7.14

[a] Data for the nonterminal residues X in tetrapeptides GGXA, pH 7.0, 35°C [from Bundi and Wüthrich (1979a), except that more precise data were obtained for Leu, Pro, Lys, Arg, Met, and Phe using new measurements at 500 MHz].

From: Kurt Wüthrich's Book

How Much Sample is Needed?

On a good but not necessarily-state-of-the-art instrument the amount of samples needed to acquire a decent 1-D spectrum can be roughly estimated (assuming liquid NMR involving fairly small molecules):

^{1}H NMR: 0.55 ml of solution in which the solute of interest is present in at least 0.1 mM (1/2 hour run)

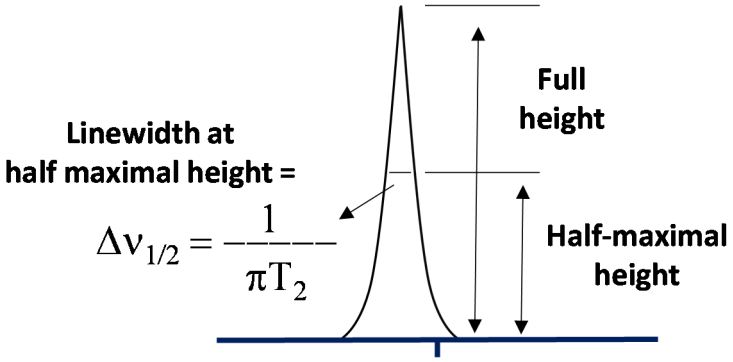
^{13}C NMR (unlabeled sample, ^{13}C is only 1% natural abundance): 0.5 ml, 1 mM will require an overnight acquisition

^{31}P NMR: (^{31}P is present at nearly 100% natural abundance): 1 mM will yield a respectable spectrum after ca. 1/2 hour.

Because the state-of-the-art instruments available to our lab at Vanderbilt equipped with very high field magnets and high-sensitivity cryo-probes, smaller amounts of sample are required. *Indeed, we have also observed for salty (normal) aqueous samples that 180 ul samples in 3 mm tubes give essentially the same signal-to-noise as 5 mm samples with equal protein concentration.* So, unless doing a titration or some other experiment where a 5 mm sample might be preferred (0.55 ml or higher sample volume), we will use 3 mm tubes, which require only 180 microliters. Protein samples in the range of 0.1-0.5 mM are typically sufficient for acquiring both 1-, 2- and even 3-D NMR spectra.

Line Widths

As suggested above, the spectral resolution (the degree to which different nearby peaks can be distinguished from each other) depends on the line width. In classical NMR jargon "line width" is defined as shown:



T_2 is called the "transverse" or "spin-spin" *relaxation time*. For a given type of nucleus, this time is determined primarily by the mobility of the molecule in solution. Highly mobile species have long T_2 relaxation times and very sharp peaks. Since there is usually an inverse proportionality between molecular size and mobility (see Stokes-Einstein equation), the following relationships usually hold:

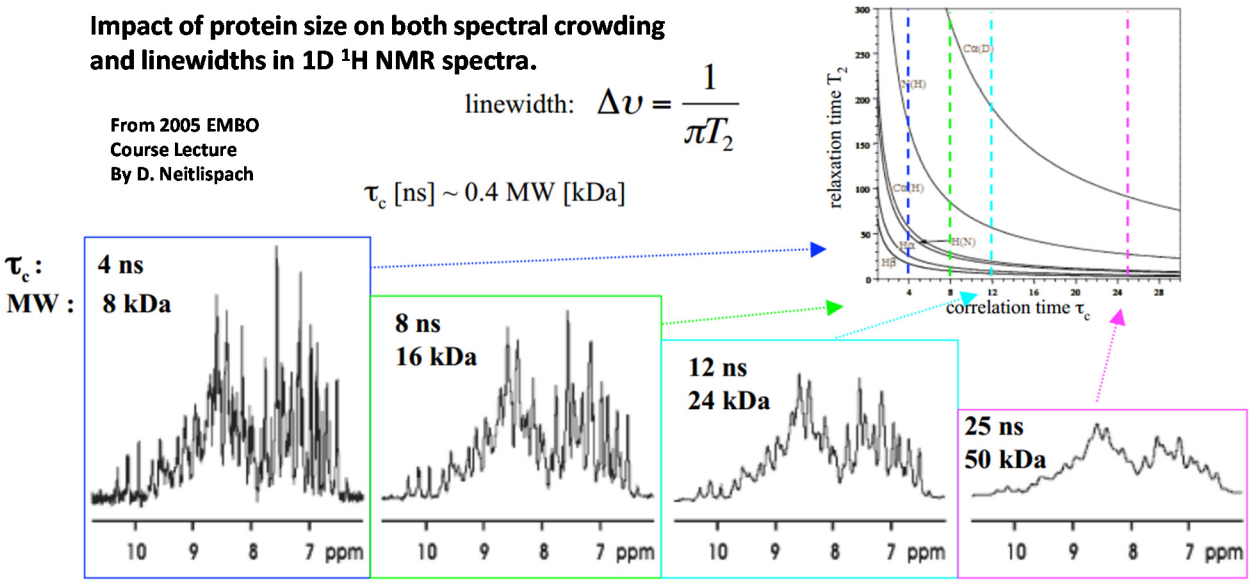
small molecules → rapid tumbling → long T_2 → sharp peaks

big molecules → slow motion → short T_2 → broad peaks

In fact, there are quantitative expressions (equations) that relate motion to T_2. Careful analysis of relaxation data (including T_1 data, see below) can shed considerable light on molecular dynamics (motions).

T_2 relaxation also determines how fast the raw NMR data, the “free induction decay” (FID), decays to zero

(where there is only noise). As indicated above, a short T_2 means the FID will decay to all-noise rapidly yielding broad peaks in the spectrum.

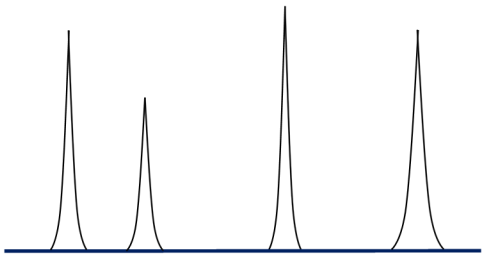


Note that magnetic field inhomogeneity across the sample also can contribute to peak broadening, a very unwelcome phenomenon. It is therefore crucial that the magnetic field be as homogenous as possible. The requirement is quite strict and we need a homogeneity of $1{:}10^{10}$, e.g. 0.1 Hz in a field of 900 MHz. Optimizing field homogeneity around the sample is achieved by a process called "shimming". During that process, the current in auxiliary coils positioned around the probehead (where the sample is) can be adjusted so that the homogeneity inside the sample is optimal. Fortunately, this process is usually now automated so that only modest intervention by the user is required.

<u>T_1 Relaxation and How an NMR Experiment is Really Carried Out</u>

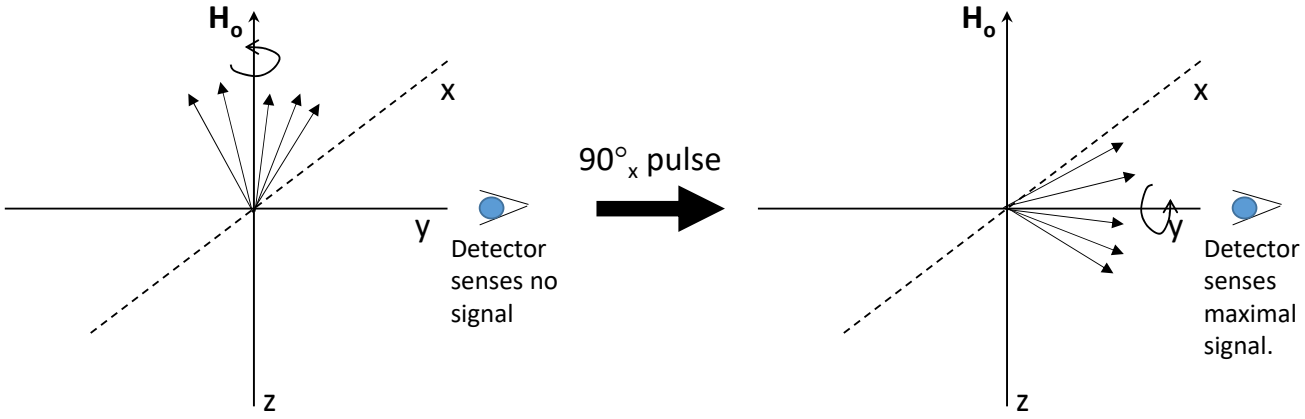
A second type of relaxation is called T_1 (spin-lattice or longitudinal) relaxation. This type of relaxation doesn't directly manifest itself as a parameter in a standard NMR spectrum, but is very important in the actual NMR experiment. To see why, let us consider how NMR data is collected.

Consider a simple ^{1}H NMR spectrum that looks like:



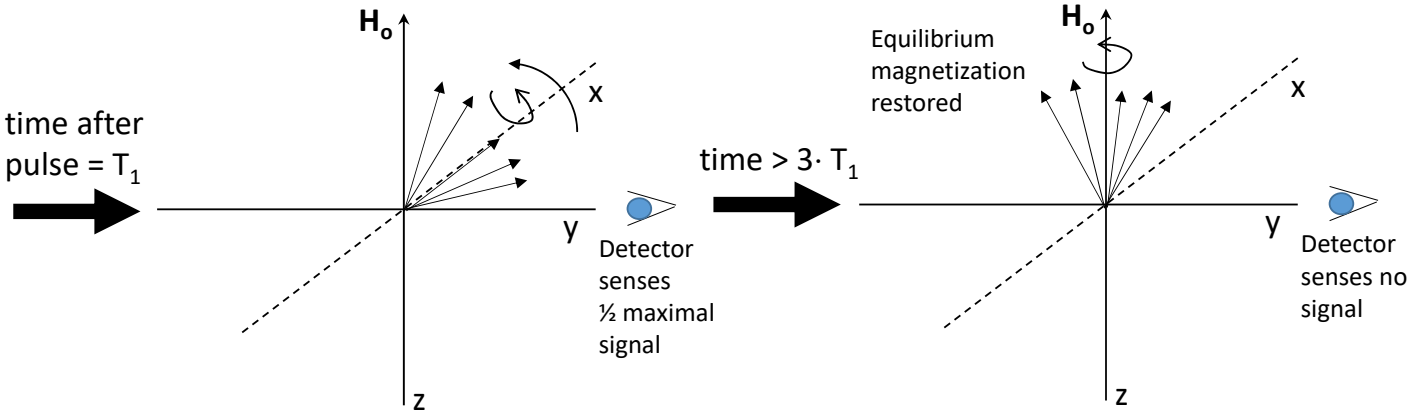
There are 4 distinct resonances, each with its own characteristic frequency. In the old days of NMR (the pre-Fourier transform days) an NMR spectrum was acquired very much like one now acquires a UV spectrum: one would pump RF radiation of a certain frequency through the sample and then gradually increase the frequency up through the full range in which absorptions are anticipated to occur. Simultaneously, a detector would sense the absorption of energy at given wavelengths or frequencies (resonance).

A modern NMR experiment is different. To understand how such an experiment works, it helps to lay aside quantum mechanics for a moment and adopt a classical picture. The 4 resonances of our hypothetical sample can be treated as four nuclear "spins" which can be represented as vectors that precess (rotate about) the direction of the magnetic field with frequencies ν_1, ν_2, ν_3 and ν_4. We will assign the field direction as the z-axis in a Cartesian coordinate system. These spins will remain aligned in their low energy equilibrium state along the z-axis(magnetic field) unless excited by RF energy corresponding to their frequencies (a phenomenon reference to as “resonance”)..



In modern pulsed NMR spectroscopy one does not excite the spins one at a time by ramping the ν of the radiofrequency (RF) transmitter. Instead all four spins are “blasted” at once with a broad band x-axis RF pulse creating a secondary magnetic field (H_1) that is orthogonal (90 degrees with respect) to the main field direction (z-axis). An x-axis pulse rotates the magnetization around the x-axis toward the y-axis (see above). "Broad band" means that the RF pulse includes a wide range of frequencies (more than spanning the range of covered by the spectrum of interest). "Pulse" means applying a very short (usually μsec) burst of high power RF energy covering a frequency range large enough to simultaneously excite all resonances present.

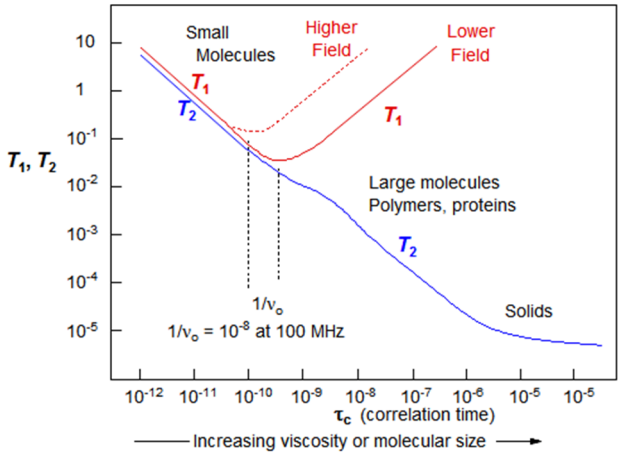
If the length of the x-pulse is chosen correctly, it is possible to rotate the all 4 spin vectors exactly 90° around the y-axis, directly into the path of the detector located along the y coordinate axis. The detector senses the time-dependent (frequency dependent) oscillation in energy caused by the 4 processing spins, each at its characteristic frequency. The detector only senses the components of the vectors that are along the axis of the detector.



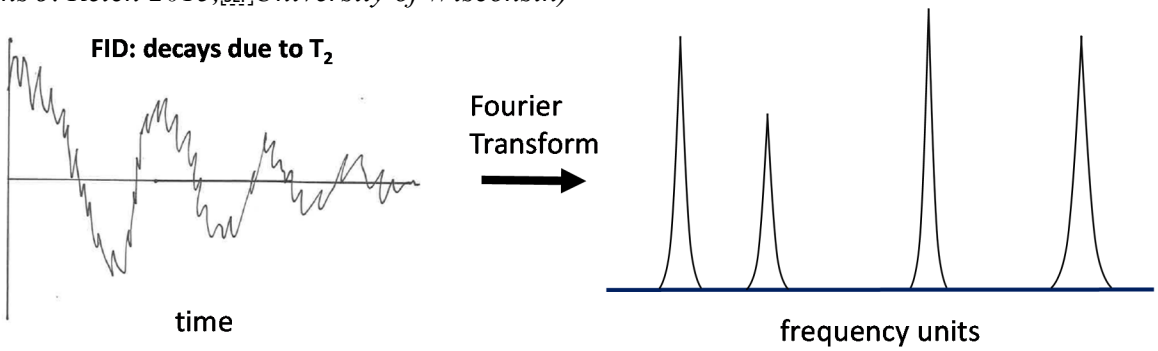
Following the $90°_x$ pulse the spins do not stay indefinitely in their high-energy "excited" states. Instead they will begin to "relax" back towards equilibrium. This is tantamount to a rotation of vector processing around the detector axis (y), 90° back to the field axis (z). During this period the detector stays on, and as long as there is still a vector component along the detector axis, it can sense the ν-dependent precession. The relaxation back to

equilibrium is known as T_1 (spin-lattice or longitudinal) relaxation and is characterized by a half-time constant of T_1. T_1 values for molecules in solution and I=1/2 nuclei are typically in the range of milliseconds to a few seconds.

The total cycle of pulse-then-detect is referred to as a single NMR "scan". What does the detector "see"? It observes an induced voltage change, reflecting the oscillating radiofrequency field as a function of time. Superposition of the four frequencies creates a complex waveform containing all 4 population-weighted frequencies. This oscillating detected field decays for each of the 4 spins. As noted earlier this "raw data" is called a "free induction decay" (FID). The decay of this oscillation in bio-molecules is due to T_2 (transverse) relaxation. T_2 is always equal or smaller than T_1 for any given spin.



The Figure above shows the behavior of T_1 and T_2 as a function of the correlation time, τ_c , which is the time it takes the average molecule to rotate in solution by one radian *(adapted from Bloembergen, E.M. Purcell, R.V. Pound "Relaxation Effects in Nuclear Magnetic Resonance Absorption" Physical Review **1948**, 73, 679-746) (Figure Hans J. Reich 2015, University of Wisconsin)*



NMR spectra are usually acquired by collecting many scans and signal averaging their FIDs. The signal-to-noise (S/N) ratio of the final spectrum is proportion to the (# of scans)$^{1/2}$. (The more the better. But note that the number of scans must be increased by a factor of 4 to increase the signal-to-noise by a factor of 2!) T_1 is important to keep in mind, because if the magnetization has not returned to equilibrium following a pulse ("not fully relaxed"), application of a second pulse will result in (very qualitatively) *over*-excitation, partially saturating the signal which leads to a loss of signal intensity. Ideally, one should wait at least 5 x T_1 between scans to have 99.3% of the magnetization in equilibrium, which can significantly increase the time needed for an experiment—particularly when many scans are required to obtain an acceptable S/N ratio. Practically it has been shown that the optimal time efficiency lies at 1.3 x T_1, where the experiment time balances the saturation effect in an experiment where 90° pulses are required, as in almost all of our experiments (*J. Mol. Spectrosc.*

1970, 35, 298-305).

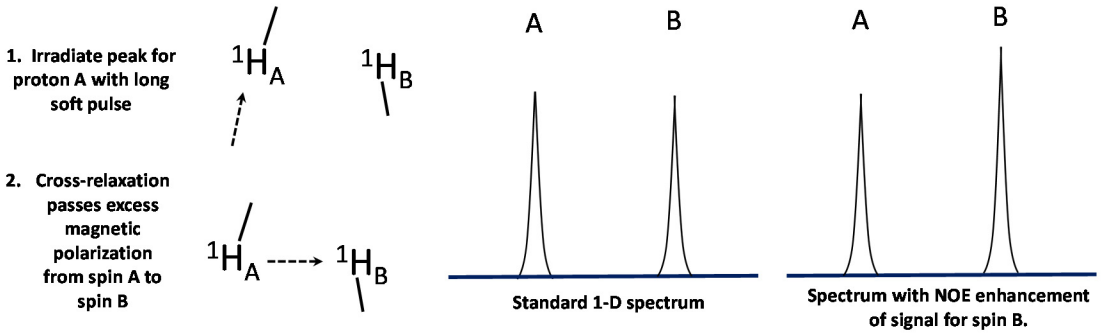
The raw data of an NMR experiment is *time* vs. the signal-averaged intensity (FID) data. What ultimately is sought is *frequency* vs. intensity data. It is possible to convert "time domain" data into "frequency domain" data by applying a mathematical operation to the data called the Fourier Transformation. The FT converts the complex waveform of an FID into the component peaks of the final NMR spectrum.

Other Relaxation-Related Stuff

In the above discussion, you may have at least gotten an intuitive feel for one type of relaxation. There are other relaxation-based phenomena that are of considerable utility to biochemists. These include the following.

1. The Nuclear Overhauser Effect (NOE)

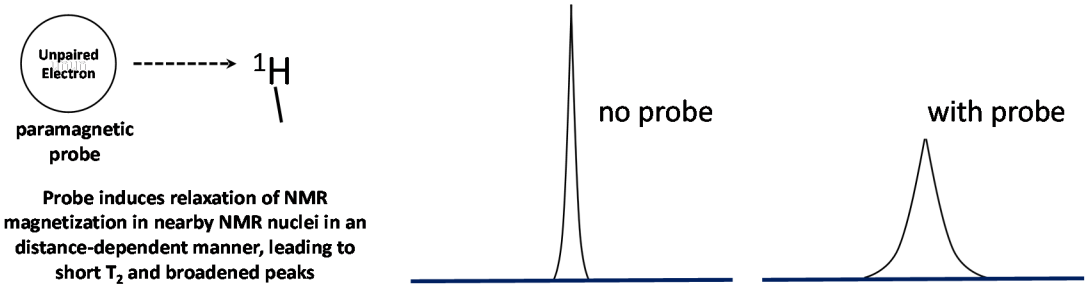
When two spin-1/2 nuclei are close to each other in space (< 6 Å), magnetization resulting from excitation can sometime be transferred from one nucleus to another. The physical mechanism by which this occurs is often referred to as "cross-relaxation". This phenomena leads to a simple but very useful experiment:



The degree to which the intensity of B is increased as a result of specific irradiation at A is a function of the distance between the two interacting nuclei (enhancement is proportional to $1/r^6$). Thus, NOE measurements allow determination of the distance between specific nuclei. This is very useful. Consider a protein. Knowing a single distance can put a lot of "restraints" upon possible 3-D structures. For protein NMR we now rarely measure NOEs 1 at a time in a 1D experiment, but instead measure numerous NOEs in a single 2D or (usually) 3D NMR experiment. More on this later.

2. Paramagnetic Probe-Induced Relaxation

If a molecule contains an unpaired electron (many metal ions or “spin labels” such as stable nitroxides), it is said to be "paramagnetic". When a paramagnetic species gets anywhere near an NMR active nucleus, it will greatly enhance the T_2 and T_1 relaxation of that nucleus in an NMR experiment, causing shorter T_2 values. This leads to significant line broadening and results in loss of peak intensity in the spectrum of the affected nucleus. Remember, in theory, the area of the peak remains constant and is proportional to the signal it represents, hence a weaker peak must be broader to maintain the same area.

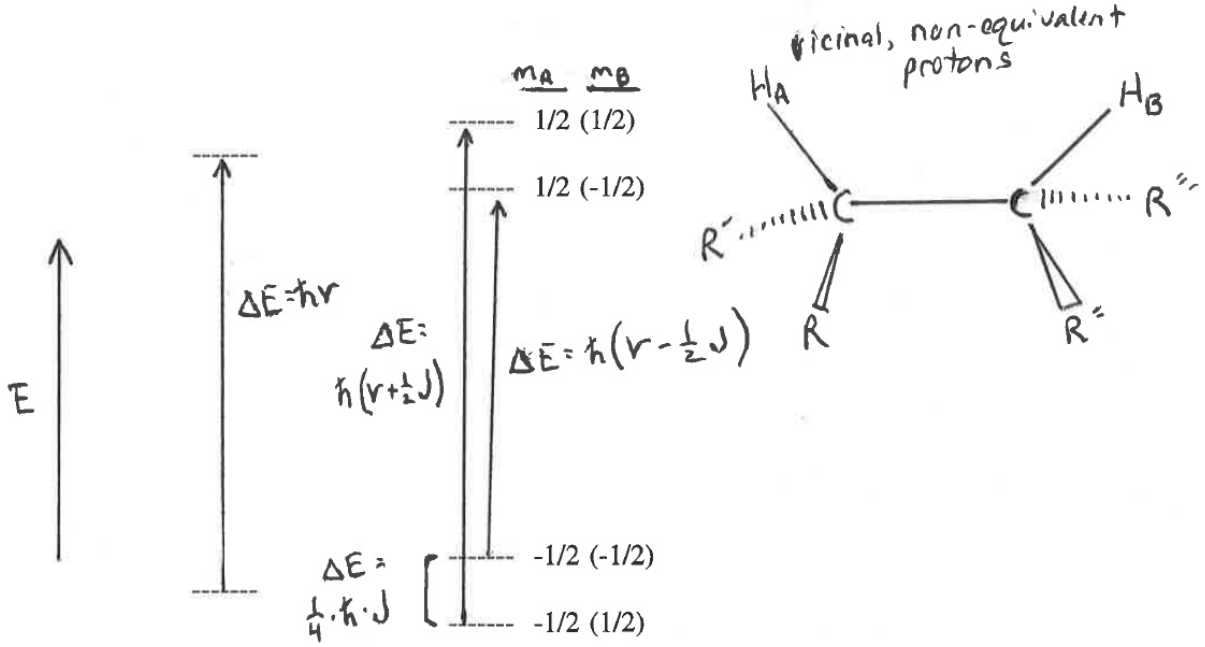


These changes (in T_1 or T_2) can be measured and are a function of the distance between the paramagnetic species and the nucleus. Such measurements can, like NOE measurements, be of considerable use in determining 3-D structures. They are also used in topology-mapping experiments for membrane proteins.

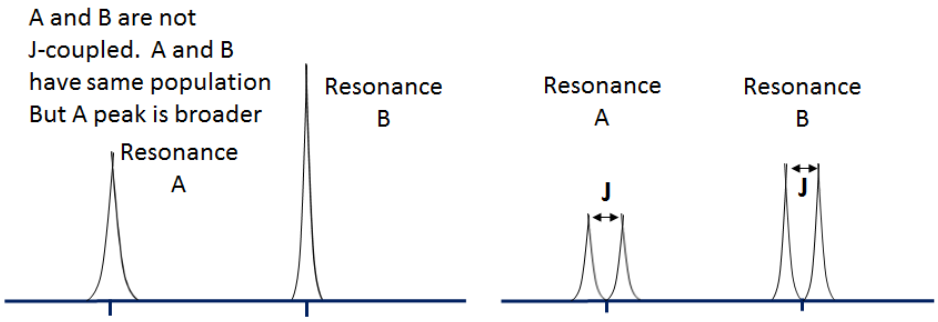
Scalar Coupling

We have now examined some of the basic features of the simplest NMR experiment and data. We must cover at least one more of them.

So far, (except for the NOE experiment), we have been talking about NMR resonances from multiple individual nuclei as if they were independent of each other (in our example about T_1 relaxation, we had 4 distinct resonances which did not interact). However, let us consider the case where we have two non-identical ^1H attached to a pair of bonded ^{12}C (NMR-inactive) atoms ("vicinal" protons). Recall that for a single nucleus there will be two possible energy states and a single transition associated with a single resonance frequency. However, if there are two protons near to each other, each can sense the quantum state of the other's magnetization. Thus, for either nucleus, there will now be *four* energy states because each original state will be split into two states based on m=1/2 or m=-1/2 of the other nucleus. The amount by which the new states are different from the original single spin state is given by $E = 1/4 \cdot \hbar \cdot J$, where $\hbar$ is Planck's constant and J is the "scalar coupling constant". So, instead of each ^1H having a single resonance from the -1/2 to 1/2 transition, there will be two transitions with energies differing by $E = \hbar \cdot J$, corresponding to the -1/2 (1/2) to 1/2 (1/2) and -1/2 (-1/2) to (1/2) (-1/2) transitions. This same phenomenon will apply to the energy transitions of the second nucleus as well.



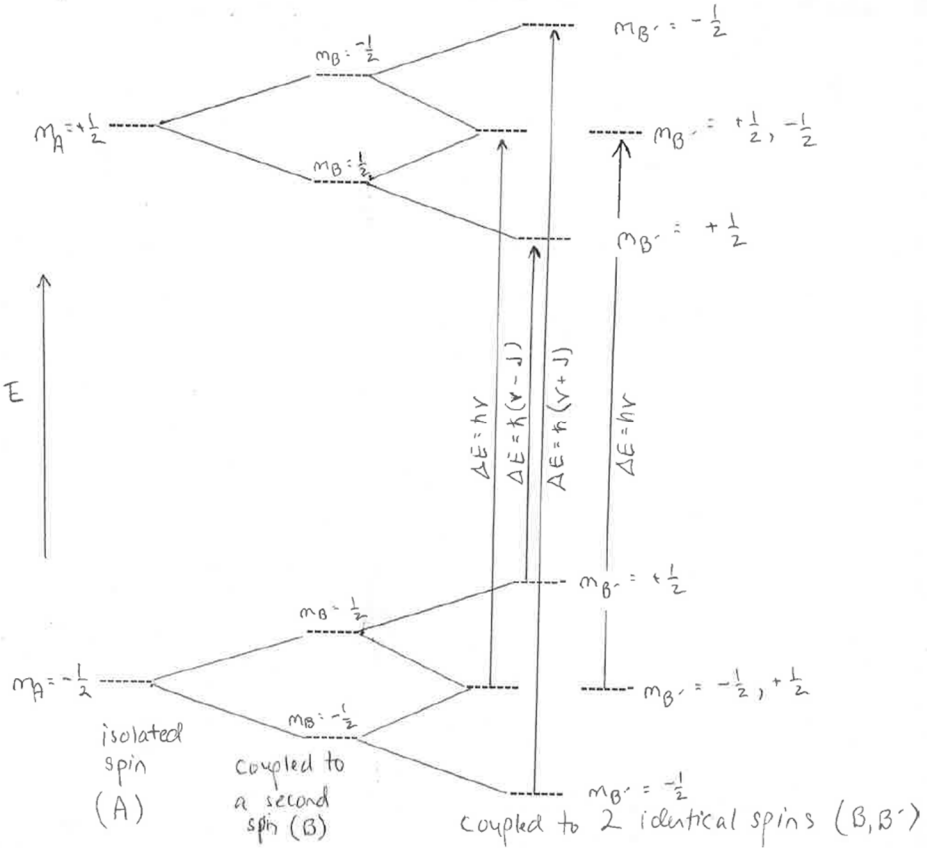
In a case such as this, the nuclei are said to be "coupled" and give rise to the following:



J is known as the "scalar" coupling constant and gives the separation ("splitting") of the peaks in Hz. In this case since the resonances from each species are split into two, the resonances of each are known as "doublets".

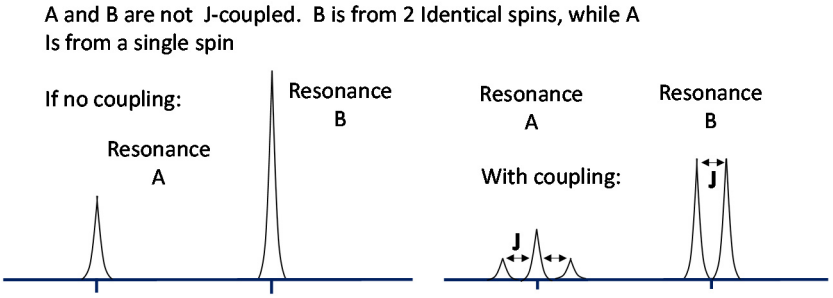
When are spins within a molecule scalar (J) coupled? Coupling is a "through bond" behavior because the spin state of one nucleus is detected by another as a result of the perturbation of the electric field of the other. Thus, to be J-coupled, there must be a covalent bonding route connecting the two spins. In addition, the size of the J coupling is usually dependent upon how many bonds are separating the two coupled spins. In the case of protons, couplings are usually observed for vicinal protons (separated by three bonds) and for stereochemically distant germinal protons (separated by only 2 bonds), but usually not between protons separated by more than three bonds. Coupling is not observed between *magnetically equivalent protons*. J-coupling is also significant between ^{1}H that are directly bonded to ^{15}N or ^{13}C.

What is magnetic equivalence? Very roughly, nuclei will give rise to the same resonance and will not exhibit coupling if they are chemically equivalent. For example, the three protons of a methyl group are chemically indistinguishable and therefore do not couple to each other.



What if there are more than 2 nuclei involved in a coupled spin system? Let's look at the simplest possible case where 1 proton is vicinal to 2 magnetically equivalent protons. The 2 magnetically equivalent protons will

appear as a “doublet” peak since they will be coupled to a single proton. What about the single proton? Let's look at its energy states (above). The allowed transitions are shown above. You can see that now instead of there being two transitions having two different energies (differing from the original transition by ± ħ·J/2), there are now three transitions corresponding to energies ħ·ν+J, ħ·ν, and ħ·ν-J. The middle transition can occur by two different routes and therefore twice as probable. Thus, now you would get a spectrum with 1 doublet and 1 "triplet". The rule is: for every n identical spins coupled, the multiplicity is n+1. The total integral (area) of the doublet peak from the 2 identical protons will be twice that as the integral of the triplet peak from the lone proton.



For the case of a single spin coupled to 3 identical spins (a in a methyl group), a quartet with component intensities of 1-3-3-1 would result for the single spin coupled to the methyl protons.

What if a single spin is coupled to two *non-identical* spins? In this case the single spin will experience coupling to both spins with non-equivalent J values (you can prove this to yourself with an energy diagram if you like) and the peak from the single proton will appear as a "doublet of doublets". I think that you can extrapolate to more complex cases. (doublet of doublet of triplets, etc.)

Here are some typical ranges of scalar coupling constants:

^{31}P-O-^{31}P	10-30 Hz	^{1}H-C-^{1}H	10-20 Hz	^{1}H-C-N-^{1}H	0-15 Hz
^{1}H-^{13}C	100-200 Hz	^{15}N-^{1}H	85-100 Hz	^{1}H-C-C-C-^{1}H	near 0 Hz
^{1}H-C-^{13}C	0-10 Hz	^{13}C-^{13}C	30-60 Hz	^{1}H-C-C-^{1}H	0-15 Hz

The presence of scalar coupling can be very useful. It is a key parameter used in chemical analytical NMR to help determine the covalent structure of a molecule. The phenomena is also central to many 2-D and 3-D NMR experiments and, as such, is crucial in the determination of the 3-D structures of biopolymers. For vicinal protons, the size of the coupling constant is a function of the dihedral angle between the two protons (the “Karplus relationship”). Scalar coupling can be used in conformational analysis of small molecules and relative small proteins and nucleic acids.

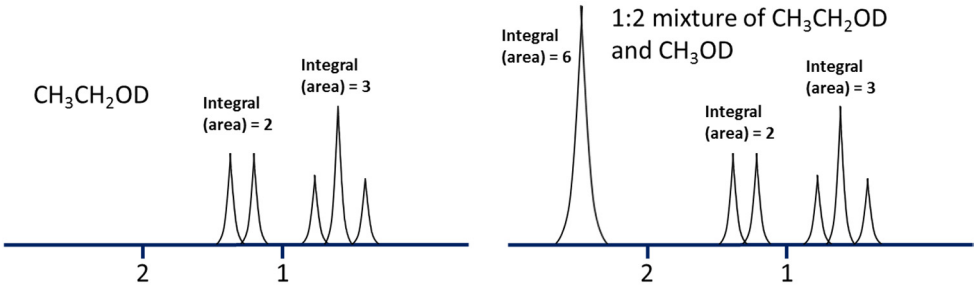
Finally, it should be pointed out that when the difference in resonance frequencies between two coupled spins is of the same order of magnitude as J, the coupling pattern can be very complex: this is what is known as "strong" or "higher order" coupling. Interpretation of such spectra can be challenging.

Relative Intensities of Signals

Each nucleus has its own intrinsic "receptivity" for NMR (meaning a characteristic intensity of NMR signal at a given magnetic field). For a given nucleus type in a sample where there are several distinct spins present, the "intensity" of the signal from each spin is defined as being its *integral* (area of the peak) and will be *directly proportional* to its concentration, assuming full relaxation between scans. See the figure below.

From this example (figure below), we see that NMR can be used in the study of a pure compound to determine

how many (magnetically equivalent) protons are represented by a resonance. Also, in *mixtures* of compounds, the relative intensities of the peaks from the multiple compounds can be used to quantitate the relative concentrations of the molecules. For example, in our lab 1-D NMR is used to quantitate the relative concentrations of mixtures of lipids and detergents in membrane protein NMR samples.



Summary of Key Concepts and Parameters

Resonance Frequency: each nucleus has its own intrinsic absolute frequency at a given magnetic field. The actual observed frequency is tweaked a bit from this intrinsic frequency due to the local chemical environment of the nucleus.

Chemical Shift: A magnetic field-independent measure of the relative frequencies of NMR resonances. It allows you to directly compare the resonance positions of spectra taken at different magnetic field strengths.

Spectral Resolution: Peaks whose resonance frequencies (in Hz) are separated by much more than their line widths are said to be "fully resolved". Resolution is proportional to the size of the magnet used.

T1 and T2: Relaxation times. T_1 determines the maximum scanning rate. T2 determines the line width. The values of both relaxation times are largely determined by molecular motions.

Scalar Coupling: Through-bond "spin interaction" between non-equivalent nuclei- results in the observation of "coupling" (splitting) of resonances into "multiplets".

NOE/paramagnetic relaxation: through-space magnetic interactions, which offer a route to determining intermolecular distances.

Relative resonance intensities (integrals): are directly proportional to the number of nuclei in the sample giving rise to the resonances.

Averaging in NMR

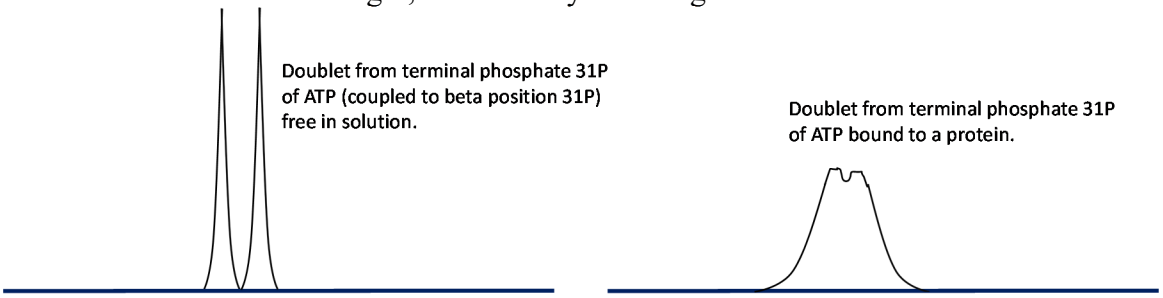
Let us consider two "dynamic" situations commonly encountered in biochemistry

$$\text{conformation 1} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{conformation 2}$$

$$\text{free ligand + receptor} \underset{k_{dissoc.}}{\overset{k_{assoc.}}{\rightleftharpoons}} \text{ligand-receptor complex}$$

In both cases molecules are interchanging between two "states" due to an exchange process.

Consider this situation from an NMR standpoint. The "intrinsic" spectra of the molecules in the two states are generally not going to be the same. For example, when ATP binds to a kinase, the ^{31}P and ^{1}H resonances for the bound ATP will appear at different chemical shifts than the corresponding resonances of free ATP. Also, the resonances of the bound ATP will be more broad than for free ATP as a result of a shorter T_2 that results when ATP associates with the much larger, more slowly tumbling kinase.

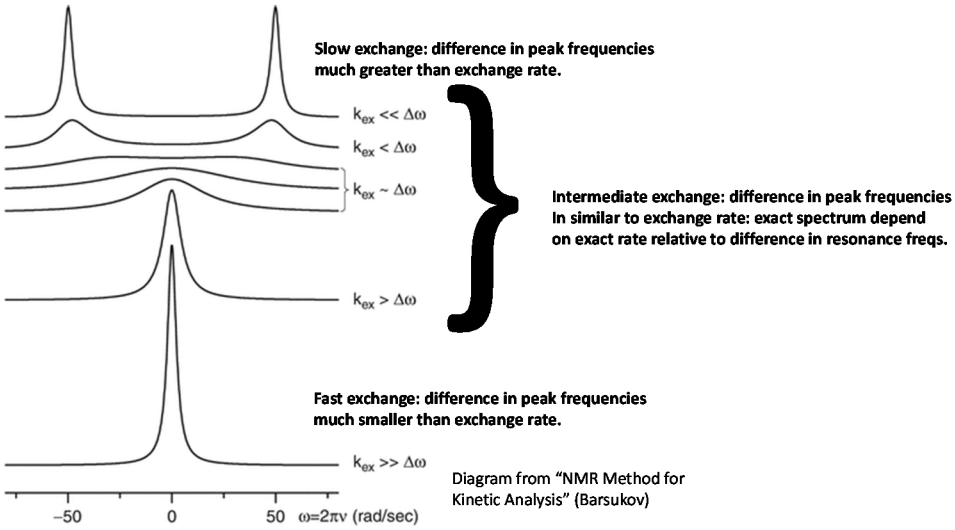


This leads to the obvious question: when there are significant populations of molecules in both bound and free states, do you see one set of peaks from the bound molecules and one set from the free, or do you instead see only a single set of average peaks from both states? The answer depends on the unimolecular *exchange rate* (k_{on}[protein]+k_{off} = k_{ex}) and its relationship between the resonance frequency difference of the corresponding resonances in the two states.

If ν_{state1} - ν_{state2} ($\Delta\nu$, in Hz) >> k_{ex} (in Hz) ("slow exchange" conditions) then you see the intrinsic resonances of *both* species.

If $\Delta\nu$ << k_{ex} ("fast exchange") then you only see a single set of signals and they are the population-weighted *average* of the intrinsic resonances.

When the exchange rate is of the same order of magnitude as the difference in intrinsic resonance frequencies, the situation is known as "intermediate" exchange and the observed spectrum will be more complex - averaging is incomplete and peaks are broadened. See example of two species (each giving rise to a single peak) below:



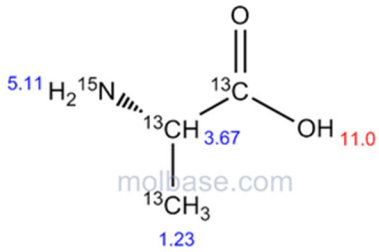
Other NMR parameters can be affected by exchange processes as well (NOE, T_1, T_2, J, etc.). For example, sometimes when a ligand binds to a protein it undergoes no change in resonance frequency, but does exhibit a

very different line width (due to different T_2 values for the bound and free states). In this case, slow, fast, or intermediate exchange behavior will be observed due to the relationship of the exchange rate to the difference in T_2 relaxation rates ($1/T_{2,off}$-$1/T_{2,on}$), as shown above.

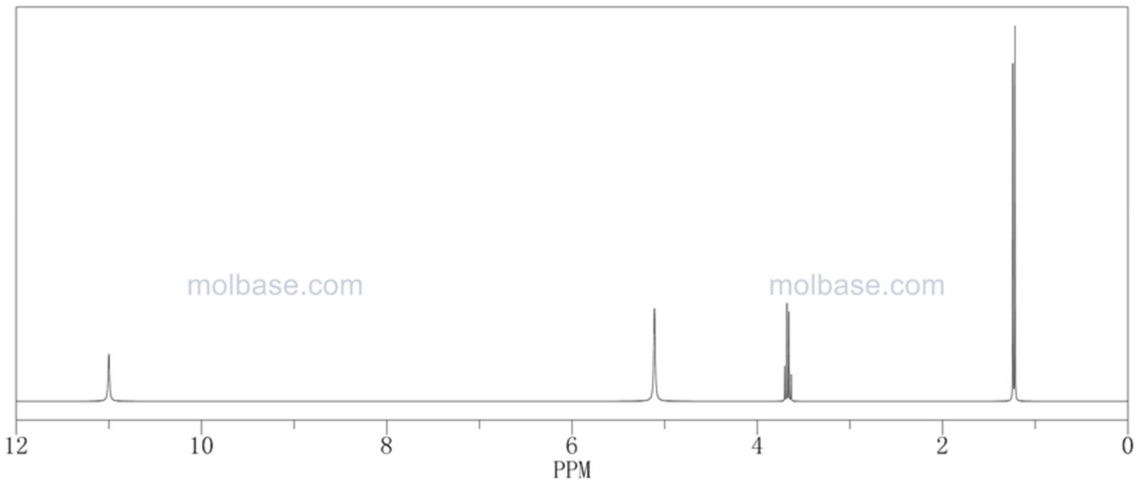
Since exchange processes are so frequently encountered in biological NMR, the above principles are very important to understand. In addition, since NMR spectra are sensitive to the presence and rate of exchange processes, NMR is occasionally used as a tool in *kinetic* (rate) studies.

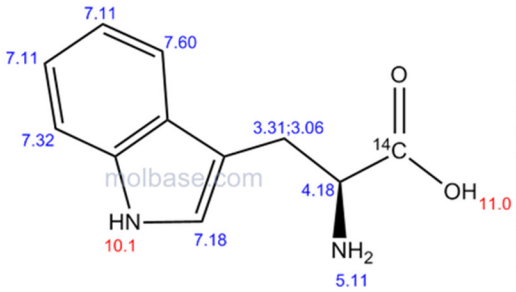
Some 1-D Spectra

We have now considered the basic features of the simplest 1-D NMR experiment and data. On the next pages are shown some illustrations.

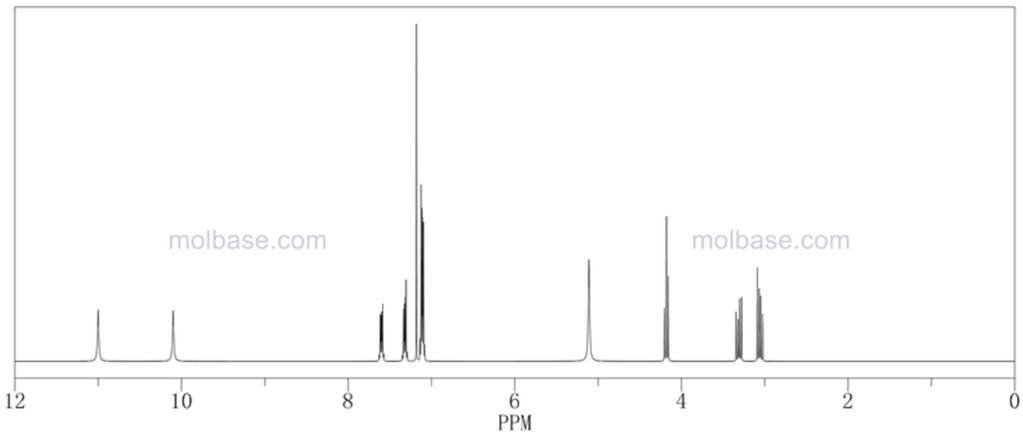


1H NMR spectrum of alanine in an organic solvent In water the amino and carboxyl 1H peaks are usually not observed because they are in rapid exchange with water (or D2O). Note that this Spectrum is of alanine that is NOT 13C-lableled. if it was labeled the peaks would be split by coupling to their directly attached 13C.



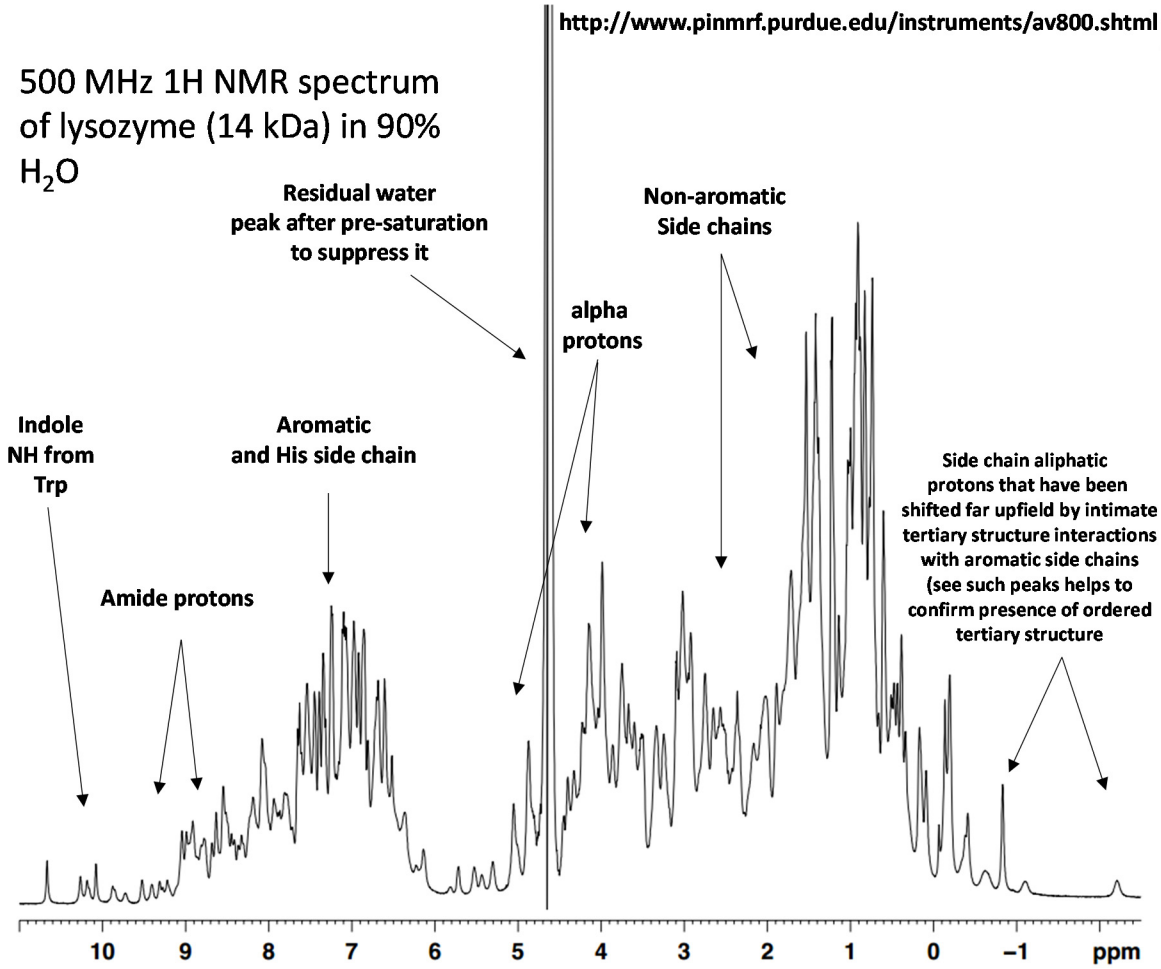


1H NMR spectrum of tryptophan in an organic solvent. In water the amino and carboxyl 1H peaks are usually not observed because they are in rapid exchange with water (or D2O). The proton attached to the indole nitrogen is seen in H2O samples because it exchanges only slowly with water (similar to the backbone amide protons). However, it will disappear in 100% D2O due to replacement with deuterium, similar to solvent-exposed amide sites.



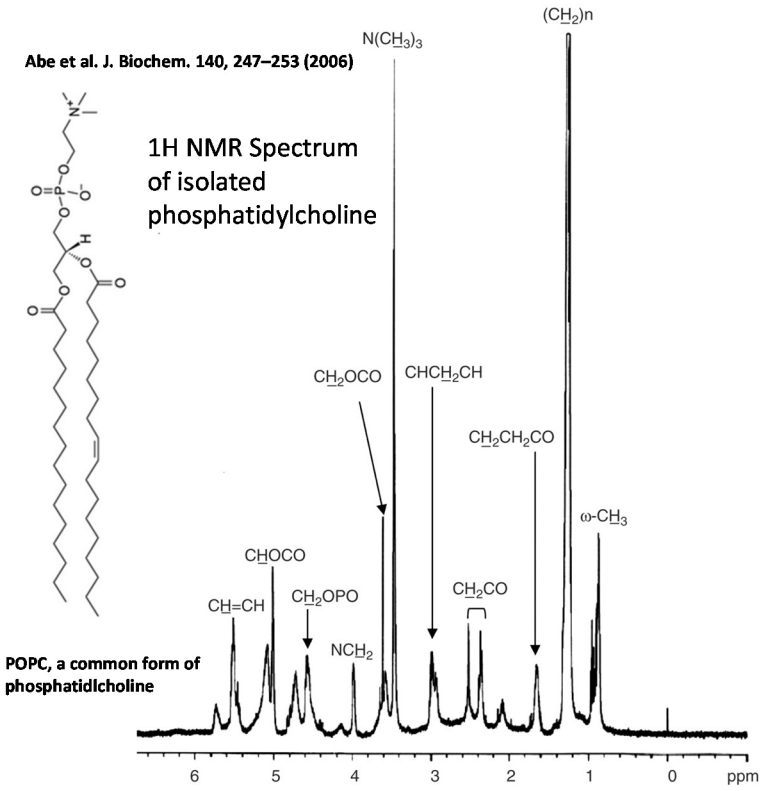
5mM lysozyme in 90% H2O / 10% D2O, ZGPR, ns = 16, DRX500-1 TBI probe.

http://www.pinmrf.purdue.edu/instruments/av800.shtml

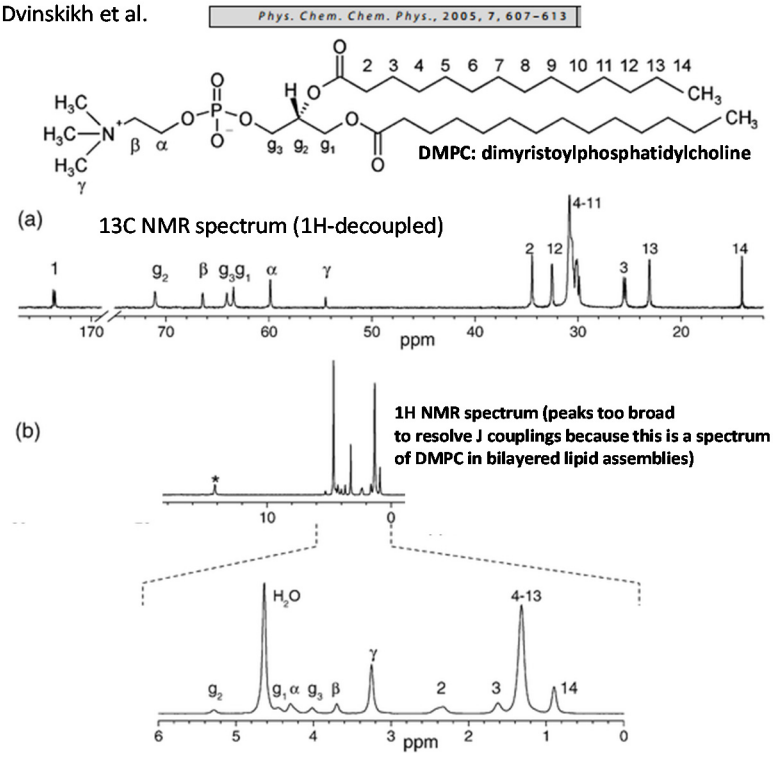


BRUKER

NAME	lysozyme-tbi
EXPNO	2
PROCNO	1
Date_	20080215
Time	15.24
INSTRUM	spect
PROBHD	5 mm TBI 1H-13
PULPROG	zgpr
TD	16384
SOLVENT	H2O+D2O
NS	16
DS	8
SWH	7507.507 Hz
FIDRES	0.458222 Hz
AQ	1.0912910 sec
RG	40
DW	66.600 use
DE	6.00 use
TE	298.0 K
D1	2.00000000 sec
d12	0.00002000 sec
MCREST	0.00000000 sec
MCWRK	0.01500000 sec
========	CHANNEL f1 =======
NUC1	1H
P1	12.00 use
PL1	0.00 dB
PL9	54.00 dB
SFO1	499.8923500 MHz
SI	8192
SF	499.8900261 MHz
WDW	EM
SSB	0
LB	1.00 Hz
GB	0
PC	1.00



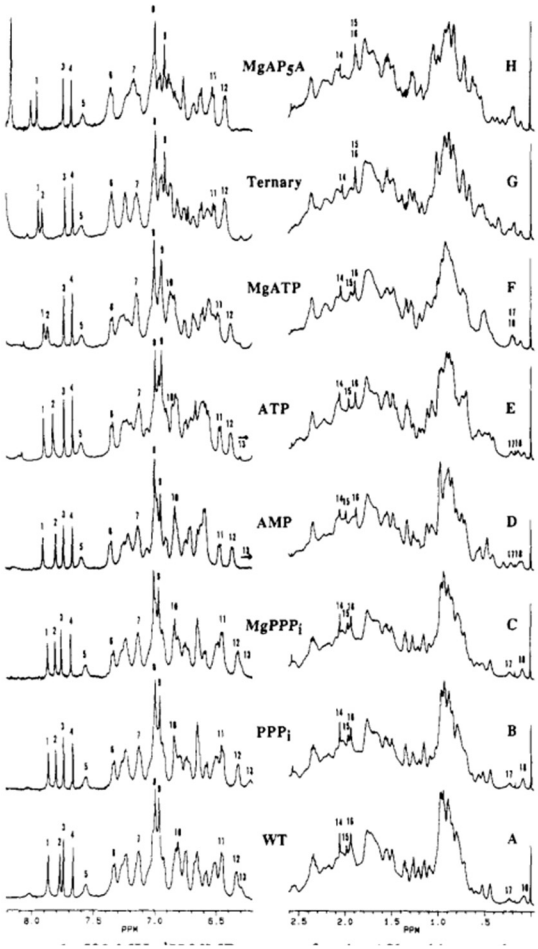
Dvinskikh et al.

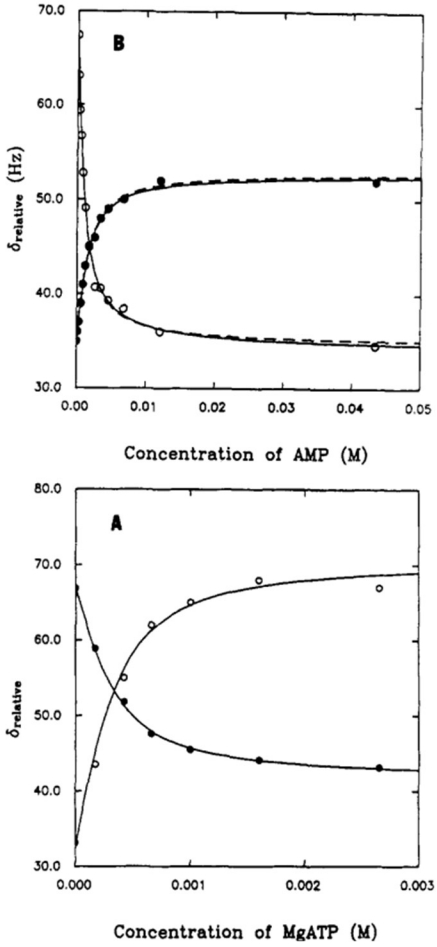


500 MHz 1-D NMR spectra of adenylate kinase (21 kDa) in D2O under conditions in which the enzyme is saturated by binding of ATP and various fragments of this ligand. Peaks 1-4 are histidine imidazole peaks.

It can be seen that a number of peaks shift in response to ligand binding. This is exploited in the panel on the far right, where the shift of peaks in response to ligand titration are used to determine the Kd of AMP and MgATP for the enzyme. Peaks 12 and 16 for MgATP, while for AMP peaks 2 and 16 were used. The free and complexed enzyme peaks are in rapid exchange on the NMR time scale.

(From Sanders et al. Biochemistry 1989, 28, 9028-9043)

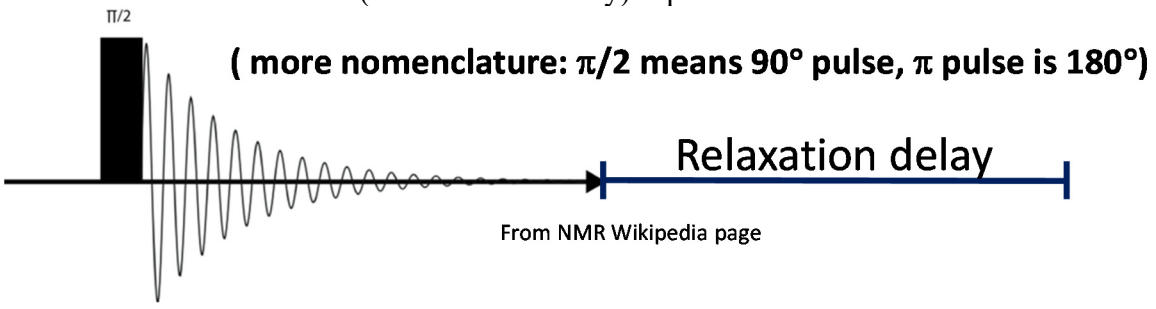




What is a Pulse Sequence?

The NMR spectra we have dealt with thus far have all been "simple" 1-D NMR spectra acquired following a "pulse sequence" consisting of a single excitation pulse (typically a $90°_x$ pulse) followed by detection (a cycle which is repeated for each scan).

(Pulse-Detect-Delay)-repeat



From NMR Wikipedia page

However, there are now dozens of more complicated pulse sequences, which allow the information provided by the NMR experiment to be tailored towards solution of particular problems. Multipulse sequences are used for:

Solvent Suppression: Sometimes the ^1H peak from the solvent (even when deuterated solvent is used) is much more intense than the largest peak of the solute of interest. This can be a real problem, especially for protein NMR, where we usually use ca. 90% ^1H_2O (ca. 100 molar ^1H!). Pulse sequences exist that allow the solvent peak to be removed or reduced without significantly perturbing the rest of the spectrum. The simplest (and quite effective) type of sequence is called "solvent presaturation". In solvent presaturation, a very weak and

long pulse is applied to the frequency of the solvent signal prior to the short high power broadband (90°) pulse. The presaturation pulse wipes out ("saturates") the net magnetization of the solvent protons, while the broadband pulse that follows excites the entire rest of the spectrum. At the time of acquisition, these signals produce a regular FID, while the solvent signals are still saturated and those spins do not contribute to the FID signal.

Decoupling: Sometimes the presence of scalar coupling can obscure information in a spectrum. Also, sometimes there is a need to identify resonances arising from coupled species. A wide variety of "decoupling" pulse sequences allows these sorts of problems to be dealt with. A very simple example is the case of ^{15}N-labeled proteins. The amide protons (attached to ^{15}N) will be doublets in 1-D, 2-D, or 3-D experiments unless the ^{15}N are decoupled from the protons when their signal is detected. Because there is usually a range of frequencies spanned by the ^{15}N that need to be decoupled "broad band decoupling" methods are used. The simplest of these methods is "continuous wave" (CW) decoupling, where the ^{15}N are irradiated with a single prolonged weak ("soft") pulse during the detection phase of the experiment. However, more sophisticated methods are typically used as part of modern 2- and 3-D pulse sequence (GARP or WALTZ, for example). These decoupling sequences use a series of clearly defined pulses for the duration of the decoupling. These sequences are usually more efficient then CW decoupling and produces less heating of the sample. In order to work properly, these pulses have to be exact, e.g. the probe has to be well tuned. There is a limit of 140 ms duration for X-nucleus decoupling on cryoprobes!

Pulse Sequences for Measuring Relaxation Time Measurements (see example below for the determination of T_1).

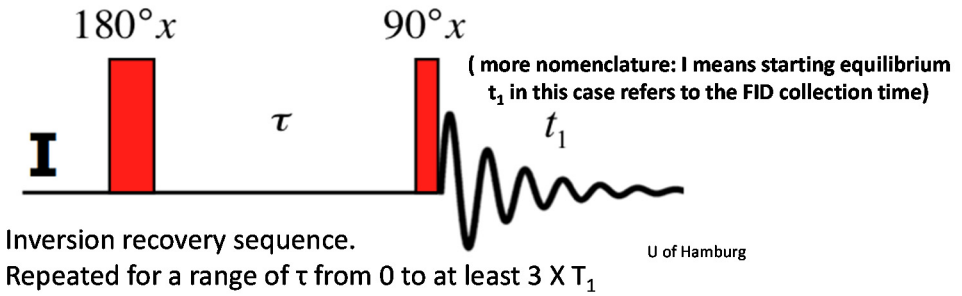
Dynamic NMR Techniques: There are a number of ways of measuring the rates of exchange processes using NMR pulse sequences. One experiment, which is showing up even in the medical literature is called "saturation transfer".

Spectral Filtering/Editing: Complicated spectra can often be simplified by acquisition using pulse sequences designed to filter out part of the spectrum (decoupling, of course, is an example).

2-D or 3-D NMR: Many of the individual techniques associated with the above are "2-D NMR" experiments. At the heart of each type of each 2- or 3-D NMR technique is a pulse sequence.

The Inversion Recovery Experiment

Pulse sequences usually involve the application of more than 1 pulse and well defined delay times before acquiring the FID. One of the simplest sequences is known as "inversion recovery" which is used to determine the T_1 values.



Let's consider a spectrum with a single resonance. The nuclear spin can again be represented as a vector precessing about an axis coincident with the magnetic field (z axis). To this "system at equilibrium" we apply a

pulse of sufficient duration to rotate the vector 180° around the x-axis so that the spins precess around the -z axis. The system immediately begins to relax back towards equilibrium and, as it does, the -z component of the vector becomes more positive, passing through the x-y-plane and (if you wait long enough) eventually reassuming its equilibrium position along the z-axis. However, what happens if another 90^0_x pulse is applied after it has relaxed only a little (at $t = \tau$), and then turn on the detector located on the y-axis? Because the magnetization of the spins now has been moved past the –z-axis into the third quadrant of the coordinate system, the detector on the y-axis "sees" an inverted resonance (negative peak), whose intensity is reduced compared to what it would be in a simple single pulse$_{90}$ NMR experiment. In fact, the intensity of the signal observed is *dependent upon how much relaxation has occurred during τ:* the observed spectrum is a function of T_1! The inversion recovery experiment is based on the following pulse sequence (also shown above in cartoon form):

$180°_x - \tau - 90°_x$ - detect

This sequence is executed for a series of τ values, resulting in a series of 1-D spectra, 1 spectrum for each τ value. Below is a depiction of the experiment and the resulting data. Note that in this diagram the individual precessing spins are not depicted. Instead, what is shown is the net magnetization in along the z and y (detect) axes for a single spin at different points in the experiment. Shown on the right are the spectra corresponding to each τ value and a plot of the peak intensities as a function of τ. You can see that the recovery of the signal as a function of τ is exponential. This data is then fit (not shown) to a single exponential equation: Peak intensity = $\text{Intensity}_{max} \cdot (1 - e^{-\tau/T1})$, where "$\text{Intensity}_{max}$" is the intensity of the peak at very long τ (which is the same as a standard spectrum after a 90° (π/2) pulse.

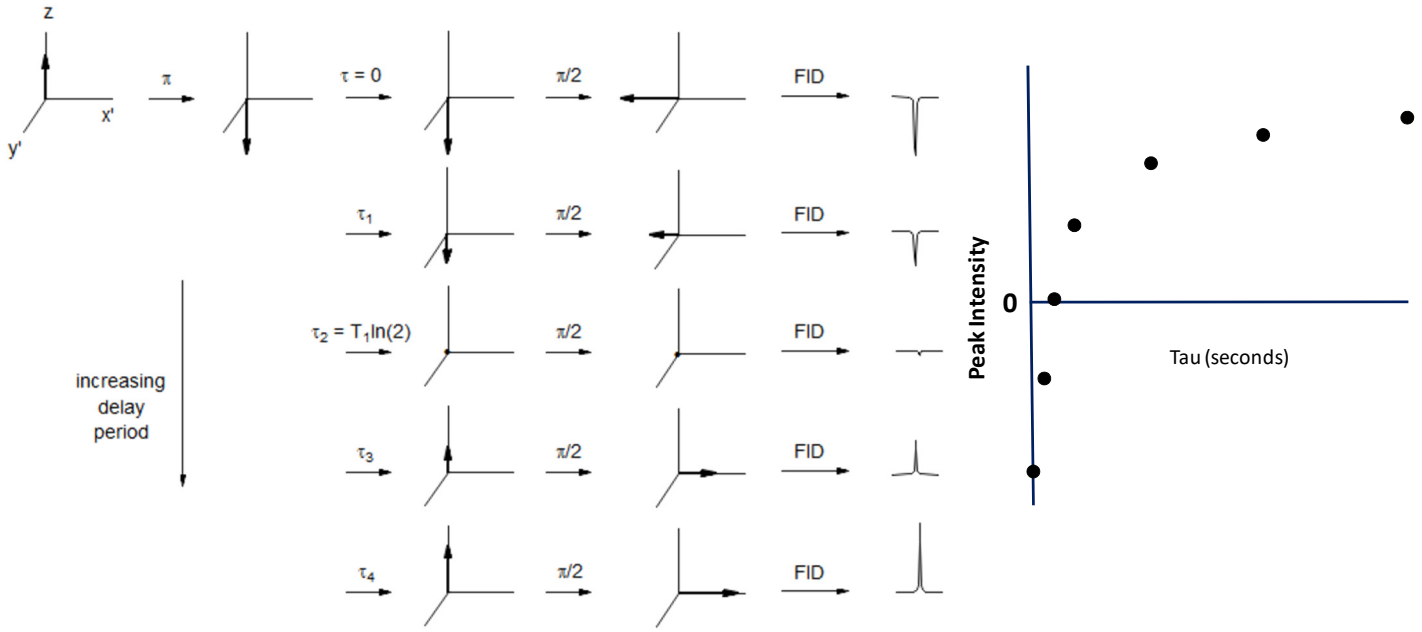


Diagram by Hans Reich, U of Wisconsin

Description of the most important parameters used to measure a NMR spectrum

From this simple example we see the principle variables of most pulse sequences:

1. *Pulse width (PW):* the length of time a pulse is applied, which is usually set to be long enough to rotate the magnetization 90° or 180° (PW_{90} and PW_{180}). The pulse width for a specific rotation (90° or 180°) depends on the power of the pulse and is hardware dependent. Pulse widths are usually in the 8-50 microsecond range for

"hard" (high power broad band) pulses used to excite spectra, but can be much longer for "soft" (low power and more selective) pulses. (PW_{90} is the time the pulse has to be applied to rotate the magnetization by 90° at a given pulse power setting. This will ½ the time of PW_{180}. The Bruker parameter is p1…p64.

Depending on their duration, pulses can be either *non-selective* (*broadband;* they excite the entire resonance range of the nucleus at once) or *selective*, meaning that the pulse is of a narrow frequency band-width, so that it excites only *selected* resonances, not all which are present. Broadband pulses are very short (μs), high power pulses, while selective pulses are long (ms) and low power!

2. Pulse Power: The pulse width required for a 90° pulse is a function of the amplifier power. This power is set high for whole-spectrum excitation pulses, but MUCH lower for selective "soft" pulses and decoupling pulses. Do not mess around with pulse power settings without first consulting the NMR facility manager or another expert. The heat generated by a high power pulse for too long of a duration can heat a sample or damage a probe. The Bruker parameter is pl1…pl64.

3. In sophisticated 2- and 3-D pulse programs, some pulses are "*shaped" pulses* that are used to selectively excite a specific section of the spectrum (usually more than a single peak and less than the whole spectrum). Usually, for a given pulse program shaped pulses and are designed and set up by advanced users—such that you don't have to worry much about how these are set up and executed.

4. The *phase* of the pulse is typically +x, -x, +y, or -y. The phase of the pulse determines whether the magnetization is pushed towards the x, y, or z-axis. In the inversion recovery experiment, 90_x and 180_x pulses were used. Remember that an x pulse rotates the magnetization in around the x axis in the direction of the y axis.

5. *Delays* are often placed between pulses. Some of these delays are fixed and are based on the need to wait for things to happen to the magnetization in your sample… such as waiting to allow J-couplings to evolve. In this example delays are set based on the size of the J (in Hz) present, using simple equations (usually given in the documentation for the pulse program). Delays can vary form a few microseconds to seconds. Other delays are incremented during the course of complex multi-FID experiments (which could be 2- or 3-D experiments).

6. A fourth variable (not illustrated by our example) is that in some experiments, multiple types of nuclei are pulsed (for example, both ^{13}C and ^{1}H), for decoupling or other purposes.

7. Most modern pulse programs further contain magnetic field *"gradients"* (usually z-axis gradients). They are applied at certain points in the program to manipulate the magnetization and the spin-spin interactions in a way that filters the final spectrum to focus on the spectroscopic parameters of greatest interest. Gradients have powers, durations (just as pulses), and can be positive or negative. For a given pulse program gradients are usually pre-defined.

8. The axis of the detector (called the "*receiver phase*") in NMR can also be switched between x, -x, y, or -y settings.

There are also a variety of additional practical settings that are key to proper implementation of any pulse sequence. For 1-D NMR experiments these include:

Sweep Width: This needs to be greater than the range of frequencies that will be included in the NMR spectrum. ^{1}H NMR peaks span about 12 PPM, so it is customary to set the sweep with to a range just larger than this (if you are working at 600 MHz and you want to have a spectral width of 12 PPM, this means the sweep width would need to be 600 X 12 = 7200 Hz). The Bruker parameter is SW [ppm] or SWH [Hz].

Filter Widths: These are there to filter out noise from frequencies outside of your spectral width. Usually these are set automatically based on your choice of SW.

Spectral Offset: This is the frequency that will define the center of your spectrum. It also called the "carrier frequency". The offset should be set to the center of where the resonances are in your spectrum (typically about 5 PPM for ^{1}H NMR). For the many pulse sequences that include water suppression the offset frequency is often set (by necessity) to be at the H_2O resonance frequency (~4.7ppm). If the pulse program includes selective pulses, these have their own offsets that may be different from the main broadband pulses. The Bruker parameter is O1…O8, where O1 is usually the primary offset.

Absolute Reference Frequency: This is the actual resonance frequency for an (ideal) unshielded nucleus in the magnet being used. If you are using a "600 MHz" spectrometer the actual proton frequency is not 600.000 MHz, but might deviates a bit from this depending on the magnet installation. The spectral offset that the user sets (see above) is the frequency relative to this absolute reference frequency setting.

Number of Time Domain Data Points (TD): This is the number of points that will be collected in your FID, which determines the digital resolution (in Hz/point) of the spectrum.

Acquisition Time (AQ): this is the time you measure the signal. Note that the combination of the sweep width setting and the number of time domain points determines how long the receiver is open to detect signal: the "acquisition time". Usually this should be longer than the longest T2 for any of the spins in your sample. If so, the signal will be almost fully decayed to near 0 before the receiver is turned off. If you "truncate your FID" (turn off the receiver while there is still detectable signal), the resonances for which the signal was truncated are said to be "clipped" and will be distorted in the final Fourier transformed spectrum. The distortions of clipping can be removed post-acquisition by massaging ("apodizing") your FID, but at the cost of artificially broadening the affected peaks.

Digital Resolution: This is determined by choice of TD and the chosen sweep width and gives the Hz/pt in the final NMR spectrum. Ideally the Hz/pt is smaller than the linewidths of the peaks in your spectrum so that peaks will be well defined, but this might not always be achievable in cases where you have fast relaxation. (Note that you can increase the digital resolution by a factor of two at no cost using "zero filling" after acquisition, as described below).

Receiver Gain (RG): This is set so that the most intense signal in your spectrum come close to maxing out the receiver (signal detector) without actually doing so. If you max out the receiver you will get distorted spectra. However if you set it too low, you will have poor dynamic range (small peaks will be noisy or not detected at all above the noise). Since we typically are working with dilute samples, the receiver gain is often set on the basis strong signals from buffer or on the basis of the residual water signal that continues to bleed through even with water suppression is used.

Type of Decoupling: The type of broadband decoupling that is turned on during the "acquisition time" (the time with the receiver is open and signal is collected) is specified by a user-defined parameter but normally set by the standard parameter set. Proton decoupling often is accomplished by a WALTZ16 sequence, while X-nuclei are decoupled by a GARP4 or adiabatic decoupling scheme.

Remember that AQ has to be <140ms on cryoprobes, if you decouple an X-nucleus during that time!

Relaxation Delay (RD): This is a delay that is applied after one cycle (pulse sequence and acquisition) before

the pulse sequence is repeated. The purpose is simply to ensure that the magnetization returns to near equilibrium before the pulse sequence is repeated. Ideally, (acquisition time + relaxation delay) should sum to at least 3 X T_1 (95% decay) for best signal intensity. However, many NMR programs are routinely excecuated under conditions where the magnetization between scans reaches a "steady state: level and does not fully relax. For these most common conditions, 1.3 x T_1 per (scan+delay) cycle is optimal for maximal sensitivity per time unit (J.S. Waugh, J. Mol. Spectrosc, 1970, 35, 298ff). For ^{1}H NMR, the total time scan time (pulse sequence-detect-delay) is usually around 3 seconds.

Number of Scans (NS): This is the number of scans that will be collected. Signal to noise increases as a function of (number of scans)$^{1/2}$. Usually (especially important for 2-D NMR) the number of scans should be set to a multiple of 4, 8, 16, or 32. This is because most pulse programs use "phase cycling" from scan to scan—optimal signal is obtained only at the end of a completed phase cycle. So, if your pulse program is based on a 16 scan phase cycle, the number of scans should be a multiple of 16.

Dummy Scans (DS): These are scans that are applied prior to the actual collection of an FID. The purpose of dummy scan is to allow magnetization to reach steady state before starting data collection. Dummy Scans are also used to reach a thermal equilibrium, which is important whenever the pulse sequence contains a power intense element, like a spinlock, decoupler or similar. 4-8 Dummy scans typically are used, but some experiments may call for more.

Temperature (TE, TESET): The sample temperature should be checked before inserting the sample. It is set and allowed to equilibrate before locking. Closely related to temperature control is air flow over the sample. Usually the air flow setting is set by the facility manager or another expert. Pulse programs can generate some heat, so there is a need to get the air flow right to make sure the sample will remain at a fixed temperature and not heat up during an experiment. Too high of an air flow can cause sample floating out of the probehead or sample vibration.

Locking: As noted above, magnetic fields of superconducting magnets drift with time, which would be a big problem for long NMR experiments. To offset any magnetic field drift, a "field frequency lock" (AKA "deuterium lock") system is used to continuously adjust the field and compensate for any drift or other disturbance affecting the magnetic field. This adjustment is based on the continuous measurement of the deuterium NMR signal from a deuterated solvent included in the sample. If the field drifts, the lock will track that drift and ensure that there are no distortions in your spectra from drift. The lock is established before shimming. Indeed, shimming is often based on using the intensity of the lock signal as a judge of quality. This is why protein NMR samples always include some D2O. Locking on the deuterium signal on a cryoprobe can be done with very little deuterated solvent (5-10% D2O is typical).

Tuning (ATMM, ATMA): After placing a sample into the magnet, it is important to "tune the probe" for each nucleus that will be involved in the experiment. This involves optimizing the radiofrequency circuit of the probe to a 50 ohm load. (The NMR manager or some other expert will instruct you on how to do this).

Shimming (TOPSHIM): The magnetic field across your sample is not completely homogeneous; there are imperfections, which means some parts of your sample can experience a slightly different magnetic field than others. This will broaden or distort your resonances. For this reason "shimming" is used to optimize the homogeneity of the magnetic field around the sample. Small coils surrounding the sample are used for this. Shimming is always carried out before starting an experiment. On modern instruments there are programs that measure the field and calculate the shim values needed to achieve best homogeneity. On long measurements (>1h), use of an autoshim algorithm is also highly recommended. This automatically and continuously re-optimizes the most important shims during the course of long experiments,

Sample Spinning: In the old days, samples were spun to help average out field inhomogeneity across the sample in 1D measurements. However, spinning can lead to its own spectral artifacts and should never be used in two- or higher dimensional acquisitions. Modern shimming equipment has eliminated the need to spin samples.

1-D NMR: Post-Acquisition Data Processing

After collecting one or more 1-D FIDs, they are saved on the computer. To generate the actual NMR spectrum the steps are:

Zero-Fill the Data (SI/TD): Because of the way NMR data is actually collected it is possible to take the original FID, which decays from time 0 to the end of the acquisition time and then add zeros to the end of the experimental data for a time that extends out to typically 2X the acquisition time. This is called "zero filling". Zero filling doubles the digital resolution of your spectrum (reduces the Hz per point by a factor of 2), at no cost.

Apodize your FID: The FID can be subjected to mathematical "massaging" functions that will alter the weighting (magnitudes) of the data points in different parts of the FID. For example "exponential multiplication" (EM: LB) increases scales up the beginning of the FID and exponentially transitions down so that the end of the FID is forced towards zero. What exponential multiplication does is to enhance the signal intensity (which is most heavily weighted near the beginning of the FID) and reduce the noise (which dominates the end of the FID). However, there is always a price to be paid and for exponential multiplication, which is that all peaks are broadened by the user-set exponential multiplication factor (if you set it to 2, all peaks get broadened by 2 Hz), which may adversely impact spectra resolution.

There are three main objectives for the use of apodization:

(1) To enhance S/N (as when exponential multiplication is used)
(2) To achieve *resolution enhancement*. Functions such as Gaussian multiplication (GM: LB, GB) will enhance spectral resolution, but at the expense of increased noise and distorted lineshapes. Note that more complicated functions such as the shifted sine bell (SIN) (in many cases squared, QSIN) (SSB) allow an optimal compromise for how to attain some degree of resolution enhancement without paying a big price in terms of signal to noise. Such functions are often critical for processing 2-D NMR data.
(3) To force any signal still present in the tail of the FID (from sharp peaks with long T_2 values) to zero, so that FID truncation artifacts are not present in the final spectrum. This is especially important for processing the FIDs of the indirect dimensions of 2-D and 3-D NMR data processing, because there almost always is signal left at the end of the indirect FIDs.

Fourier Transform (FT): This converts the zero-filled and apodized FID in to the intensity vs. frequency spectrum.

Set your chemical shift reference frequency: This is the resonance frequency of the 0 PPM standard. This can be set interactively based on picking a peaks of known chemical shift in your sample or can be set based on previous samples that contained the same solvent. In practice, this frequency can also be set indirectly by choosing a peak of know chemical shift (such as that of water at 4.7 PPM) and setting the PPM value for that peak.

Phasing (PK, APK): After FT, you will see that your peaks are usually "out of phase". You therefore manually phase the spectrum with iterative zero- and first-order phase correction until all peak in your spectrum are correctly "in phase". Note that there are two circumstances (in particular) where one or more peaks may be

impossible to phase. One case is when a peak has been "presaturated" during the pulse program (ususally the solvent peak). The other case is due to the presence of a "folded in", "folded over", or "foldover" peak. This is a peak that comes from a resonance that was outside of the acquisition sweep width in an indirect dimension of a 2- or 3- experiment (where there are no filters). When such resonances are present they usually fold into the spectrum either around the closest edge of the spectrum or appear at a frequency away from the opposite edge of your spectrum that matches the frequency that the resonance was beyond the edge of the spectrum. It can be verified that a foldover peak true is a foldover peak by changing the carrier offset—the position of a foldover peak in the spectrum will shift in the opposite direction that real peaks shift as the offset is varied. Peaks outside the sweep width in a 1D spectrum will be filtered out and don't show up on our instruments.

Baseline Correction (.BASL): Sometimes after processing and phasing your spectrum, you will see that the baseline (no peaks) is not symmetric noise along the x axis. Baseline correction can be used to correct for this to force the baseline to zero. This is very important in 1- and 2-D experiments where peak areas or intensities are going to be quantitated—for such measurements peaks must sit on a baseline = 0, not some positive or negative offset.

2-D NMR

1-D NMR spectra can be very crowded. The ^{1}H NMR spectrum of a protein may have a couple of thousand of peaks, all crammed into a mere 10 PPM. 2-D NMR allows a spectrum to be spread out in two dimensions and also enables many other useful spectroscopic tools.

Before proceeding, let us introduce the concept of "spin space". We are familiar with the concept of one's "personal space"- the realm in which we live and function, the complex matrix/network of our interactions with other creatures and inanimate nature, the workings of our conscious and our sub-conscious self. The nuclei involved in magnetic resonance have a sort of "personal world" of their own. Nuclear spins within a sample undergo a complex matrix of interactions with each other and with the magnetic field. As their environment is perturbed by intruding pulses and then allowed to evolve unperturbed, the various aspects of their personalities (chemical shift, scalar coupling, NOE, etc.) will respond in ways that are both unique to each spin and also in ways that often are interconnected with other spins and parameters. This realm of nuclear spin possibility and actuality can be referred to as "spin space".

All 2-D NMR experiments have some things in common:

There is a *direct* dimension (called "f2" or TD2 for "time domain 2"), which will always involve the nucleus that is directly detected in the experiment (most often ^{1}H). This is the dimension that will end up being the X-axis of the final spectrum. There is also an *indirect* dimension (f1, time domain 1), which is generated when the spectra from the series of spectra of directly-detected FIDs are Fourier transformed in the second dimension (see below). The indirect dimension may also be a ^{1}H dimension (as in a 2-D COSY or NOESY spectrum – homonuclear correlations), but often is a 2nd nucleus such as ^{15}N or ^{13}C (heteronuclear correlations)

A series of time domain 2 FIDs are collected. For each FID, all parameters are identical except for an incremented delay. During the 2-D experiment this delay is incremented by whole numbers of from $0\cdot\tau$, to $1\cdot\tau$, to $2\cdot\tau$, …. to $TD1\cdot\tau$, where TD1 is the total number of FIDs collected (the # of increments). At each increment this delay (often called the t1 time) will have a value of "$N \cdot \tau$" for the Nth FID collected in a 2D NMR experiment. N varies from 0 to TD1. On our spectrometers, the τ value is IN_F.

There is a Preparation Period: The preparation period is the beginning section of the pulse sequence. During this time the "spin space" of the system is manipulated to get rid of all of the spin interactions not of interest in and "isolate" (prepare) the time-dependent NMR phenomena of interest for time evolution during the t1 (also

known as the evolution period). For example in a 2-D $^{1}H,^{15}N$ HSQC or TROSY this part of the sequence isolates desired magnetization of all protons that are directly attached to an ^{15}N nucleus and purges all other ^{1}H signals. This is true even though most of the ^{1}H pulses in an HSQC or TROSY experiment excite ALL protons, not just those attached to ^{15}N.

There is an evolution period (also called t1) (IN_F): This is the t1 delay time described above, during which the spins are allowed to evolve with time in the spin space. In a 2-D experiment many FIDs are taken (equal to the number of data points for the FID in the 2nd dimension). As summarized above, n the most simple 2-D experiments, τ is incremented by an "increment time" (0 for first FID and will have a value of "N times increment time" for the Nth FID collected in a 2D NMR experiment. N varies as an integer from 0 to TD1 (TD1=total number of FIDs collected). More complex incrementation schemes of τ are required to get phase sensitive spectra in both dimensions, as required for advanced pulse programs involving States, TPPI, States-TPPI or Echo-Anti-echo schemes, to name the most important ones.

Mixing Time: During this time the pulse sequence manipulates the evolved spin state so that at the end a detectable signal is produced that is then recorded by the receiver (detector) as an FID.

Preparation and Mixing time periods contain a number of pulses manipulating the spins along with fixed delays and, often, gradients. These time periods are fixed for 2-D experiments. However, the evolution time varies according to the incremental delay, as required to generate the indirect time domain data that is the basis for the 2nd dimension of a 2-D spectrum.

To reiterate, the idea of a 2-D experiment is to take a series of spectra, each differing in acquisition only in the value of τ used during t1. The τ used are based upon an incremental delay (t1):

First FID	$t1 = 0$
Second	$t1 = \tau$
Third	$t1 = 2 \cdot \tau$
.	
.	
Nth	$t1 = TD1 \cdot \tau$

For a typical 2-D NMR experiment, TD1 is 128 to 1024, meaning that you have to take that many individual 1D spectra. Note that TD1 is usually a multiple of 32 to make sure that all phase cycling in the pulse program (in the indirect dimension) is exactly completed. 2-D NMR can take a long time! At the end of the experiment, the raw data set consists of a set of N FIDs (above). The time domain FID are Fourier transformed to give a series of spectra ("rows"). The vertical "slices" through the stacked transformed spectra (one slice for each frequency sampled) are equivalent to a series time domain FIDs in the second dimension. These slices are often referred to as "columns". Each slice (one for each data point in the first dimension) is FT'd to produce the final 2-D spectrum. Here are two illustrative figures:

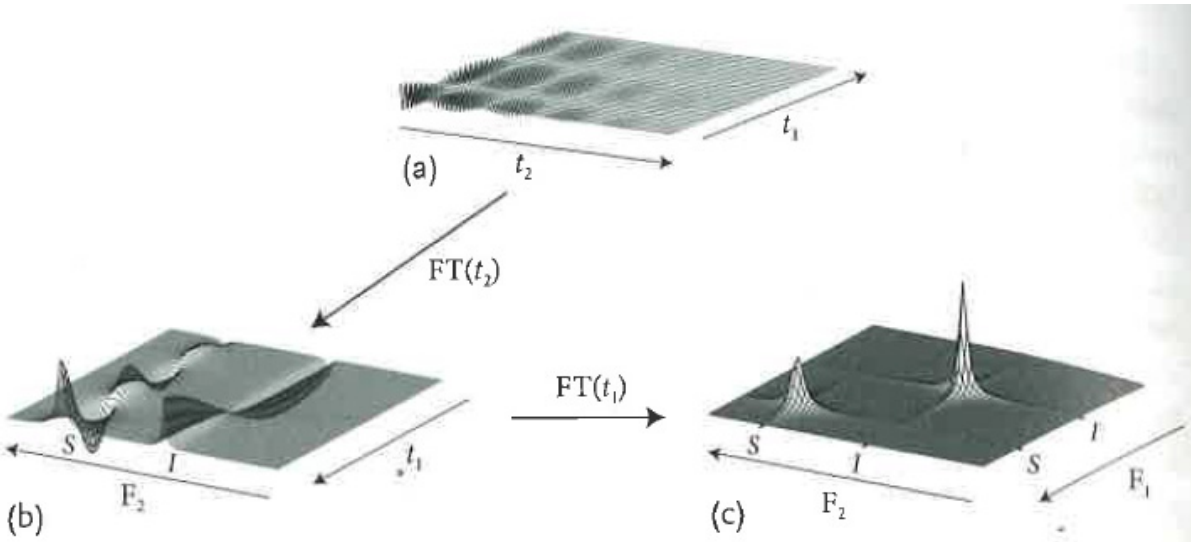


Figure 17.3 Principle of 2D NMR spectroscopy. An array of FIDs (a) is Fourier transformed, resulting in an array of 1D spectra (b), in which the signal intensities are modulated by the frequency in the indirect dimension. A second Fourier transform, this time taking corresponding data points from the different 1D spectra, results in the 2D spectrum (c). FT – Fourier transform.

From: Zerbe and Jurt, 2014

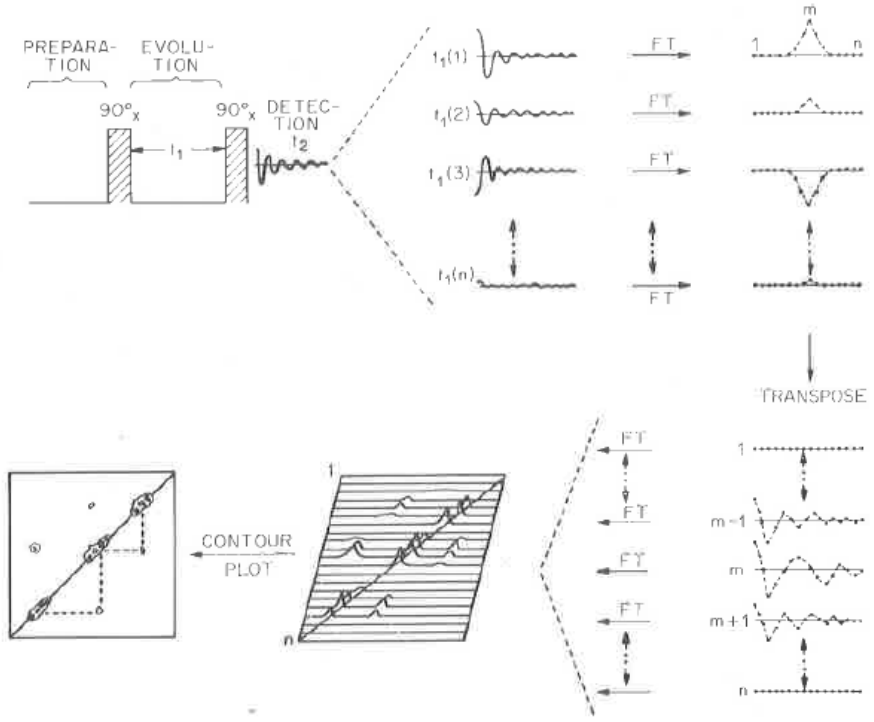


Fig. 6.1. Schematic representation of the formation of a 2D spectrum. The pulse sequence shown is actually appropriate for the development of a *chemical shift correlated* or COSY spectrum. The free induction decays and their Fourier transforms are appropriate for a single resonance, while the final spectra show two sets of *J*-coupled spins. See text.

From: Bovey, 1988

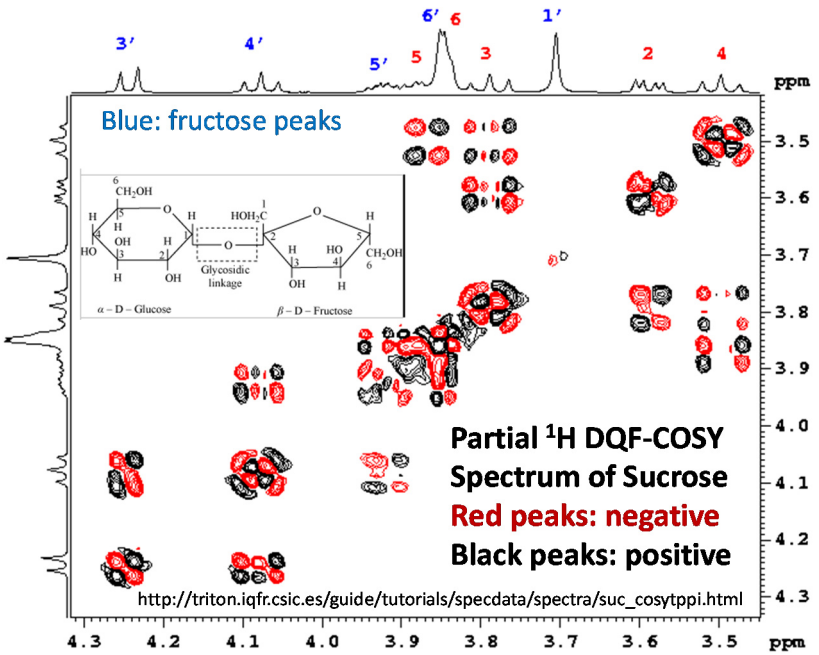
Note that in addition to Fourier transforms, processing of 2-D NMR data usually involves both zero-filling and apodization of the data in both dimensions to optimize the digital resolution, the S/N, and the spectral resolution.

Usually, 2-D spectra are depicted as a topographical contour map. Peaks can be either positive (coming out of the plane at you) or negative (dipping below the plane). For some experiments, doublet peaks are “anti-phase” meaning that one component of the doublet is positive and one is negative. In homonuclear 2D spectra, both dimension are for the same nucleus and spectra are usually mirror-symmetric around a “diagonal” which is usually the least interesting part of the spectrum, often consisting only of the original 1-D spectrum. Typically, it is the off-diagonal "crosspeaks" that are the most interesting- providing information on "what is coupled to what", "what is undergoing NOE to what" or some other useful information from spin space. In heteronuclear 2-D experiments the two dimension represent two different nuclei and there is no symmetry or diagonal to the 2-D spectrum.

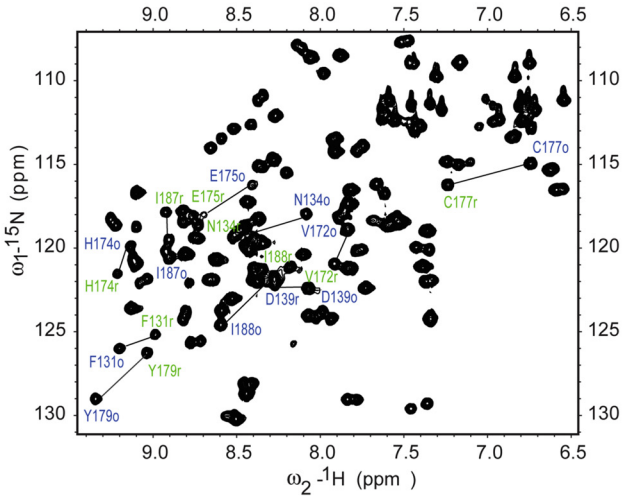
Two important parameters to consider when setting up a 2-D experiment are the number of points (number of increments, TD1) that will be collected for the indirect dimension (TD1 = number of FIDs that will be collected) and the time increment (Δ) to use for the incremented delay τ. Usually Δ is determined by TD1 and the sweep width of the indirect dimension, which needs to exceed the frequency range of the resonances present in that dimension (to avoid foldover), which is critical. Depending on the sequence and instrument the SW in the indirect dimension is $1/\Delta$ or $1/(2\cdot\Delta)$. The digital resolution (Hz/pt) of the spectrum in the indirect dimension is determined by SW/TD1 in that dimension. If the TD1 (number of FIDs collected in the 2-D experiment) is too low then you will lose resolution in the indirect dimension. However, do keep in mind that you can zero fill your data in the 2nd dimension as well and increase the resolution by a factor of 2 “for free”.

Let us briefly summarize some of the main classes of 2-D NMR experiments.

Homonuclear Correlation Spectroscopy: key 2-D experiments include COSY, DQF-COSY, TOCSY, and NOESY (through space). With the exception of the NOESY, these experiments give cross-peaks based on the J-coupled spin pairs. The TOCSY gives cross-peaks for entire J-coupled spin systems, e.g. you get cross peaks between all links of a J-coupled chain of protons, whether or not the two spin are directly coupled to each other. The spin system stops where the J-coupling between protons is missing (which for protein is usually at the peptide linkages).

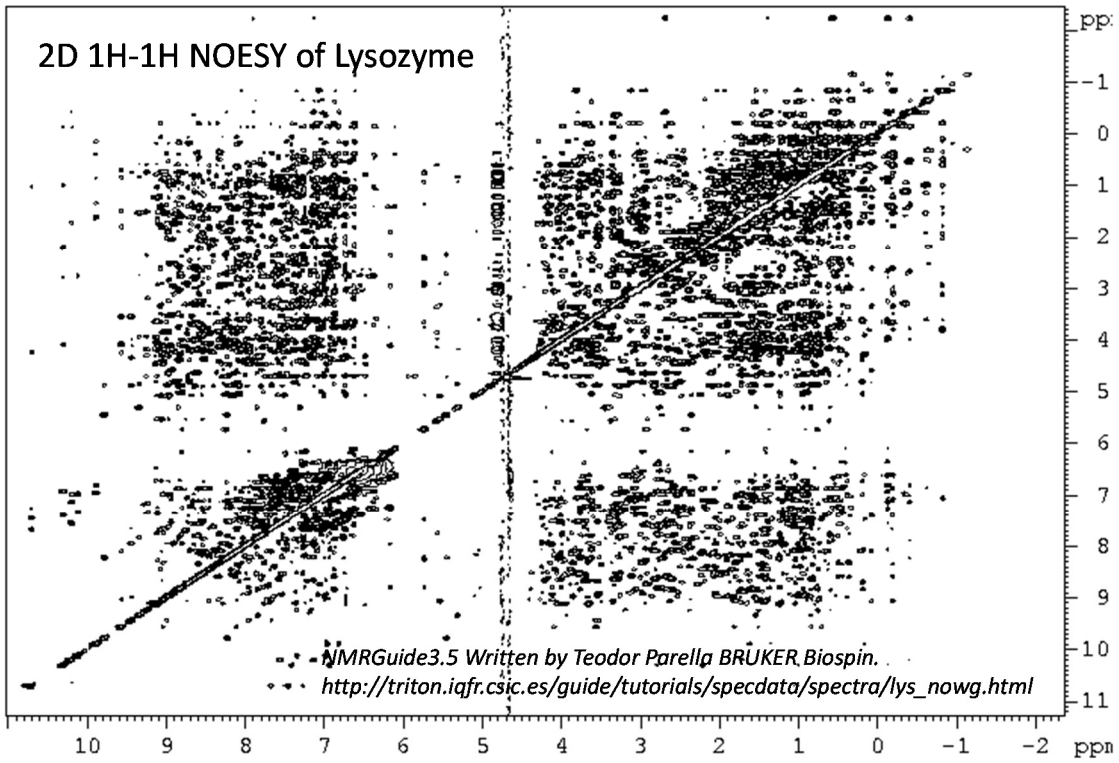


Heteronuclear Correlation Spectroscopy: key 2-D experiments include HSQC, HMQC, and TROSY. These experiments give cross-peaks between directly-detected ^{1}H and X nuclei (usually ^{15}N or ^{13}C) that are J-coupled (and most often are directly bonded). These experiments can be used as the basis for relaxation measurement experiments, usually for the X nucleus (T_1, T_2, and heteronuclear NOE measurements).



^{1}H–^{15}N HSQC spectrum of a fragment of the protein NleG3-2. Each peak in the spectrum represents a bonded N-H pair, with its two coordinates corresponding to the chemical shifts of each of the H and N atoms (mostly backbone amides). Some of the peaks are labeled with the amino acid residue that gives that signal ("peak assignments").
Bin Wu et al. PLoS Pathogens 6 (6): e1000960.

NOE Spectroscopy: (NOESY, ROESY) These are homonuclear experiments, where it is not the J-coupling, but rather the dipolar coupling between the spins that is important. This means that spins interact through space with each other producing an NOE effect. The closer together they are, the larger the NOE effect (r^6-relationship!). Just as in the correlations spectra, the 1-D ^{1}H spectrum is along the diagonal and symmetric cross-peaks (NOE-peaks) are seen that connect the diagonal peaks from pairs of protons that are close to each other in space. NOESY is used for small molecules and large molecules and complexes. The NOE peaks are positive in large molecules, negative in smaller ones. ROESY is used for medium-sized molecules (1000-5000 Da), the ROESY is used, and gives only positive NOE peaks.



Spectral Filters

As noted earlier, there are special pulse program modules found in many different pulse programs, which can be used to selectively remove unwanted parts of NMR spectra. Let us consider a couple of types of "filters" which are commonly encountered:

1. The "double quantum filter (DQF)". This can usually be incorporated into just about any kind of pulse experiment. It gets rid of the spectra from all spins, which are not engaged in scalar coupling. One of the useful things about this is that it simplifies the diagonal. For example, the DQF-COSY has an in-phase diagonal, which provides cleaner spectra than phase sensitive COSY with its anti-phase diagonal peaks.

2. "H-X" filters are heteronuclear in nature and allow removal of peaks that are not involved in heteronuclear coupling. Either nucleus can be detected in the direct dimension, but ^{1}H is usually preferred because of its great sensitivity. This type of filter is commonly used in studies of large, isotopically labeled proteins. Such filters are a key part of the HSQC and TROSY pulse programs, where the only protons that are observed are those with an attached ^{15}N.

Collecting and Processing 2-D NMR Data

The parameters used to set up a 2-D NMR experiment are very similar to those used for a 1-D NMR experiment with a few additions. For a heteronuclear experiment, the X-nucleus "carrier" offset frequency is set to an appropriate value (should be set at the middle of the X nucleus spectrum). An indirect dimension SW also needed to be chosen (which determines the τ increment of the evolution period—see above). Also needed are the PW_{90} and PW_{180} for the X nucleus in addition to the corresponding PW for ^{1}H. Most experiments that are based heteronuclear J-coupling spin interactions (HSQC AND TROSY, for example) have additional fixed delays in them that must be set based on the typical J between the protons and X nuclei under examination (about 92 Hz for amide ^{1}H-^{15}N pairs). As noted, both the number of scans per FID and the number of indirect increments (number of FIDs to acquire) should be set to be multiples of 32 (or sometimes 8 or 16 depending on the pulse sequence; multiples of 32 are always safe if you don't know).

Processing of 2-D can be done in Topspin using the XFB command. The appropriate parameters have to be set first (SI, WDW, SSB, phases and baseline – to mention the most basic ones). Alternatively, other processing software like NMRPipe is available. They often use a "processing script" that queues up zero filling, apodization and FT of all the directly-detected FIDs, followed zero filling of the columns in the direct dimension, apodization, and FT. These scripts can also include phasing and baseline correction in both dimension. The exact type of apodization and associated parameters must, of course, be set up within these scripts.

Included in the processing is setting the chemical shift reference for the 2^{nd} (and any additional) dimension. This is usually not done using internal ^{13}C or ^{15}N chemical shift reference compounds, but is instead done mathematically by relating the frequencies using in the X-nucleus dimension back to an absolute chemical shift reference setting in the ^{1}H dimension. (Talk to the NMR manager or some other expert user about this) In Topsin, the SR parameter is set to calibrate the spectrum. Caution: In the default setting (SR=0), the carbon dimension is of by about 2.5ppm!

2-D NMR: Summary

In spectroscopic terms 2-D (and higher) NMR spectroscopy is useful because:

- It spreads out crowded spectra into an extra dimension to enhance effective resolution

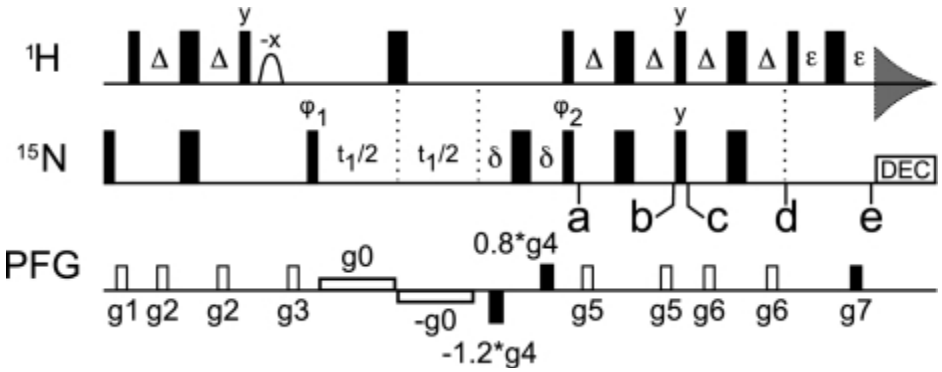
- It simplifies identification of species that are interacting with each other (via J-coupling or the NOE). This is useful both for assigning peaks and for collecting restraints for structure determination.

- It allows a selective focus on one type of interaction (e.g. J coupling, NOEs, or relaxation).

Starting in the late '80s the concept of 2-D NMR was extended to the creation of 3- and higher dimension experiments that allow the spectrum to be spread out into even more dimensions. In a 3-D experiment there are 2 evolution times (t1 and t2), each of which must be fully incremented at each value of the other (in other words, for each value of τ_1, you must acquire FIDs for the full series of τ_2). Thus, if N rows are to exist in both the second and third dimensions, N^2 spectra must be acquired (compared to simply N for 2-D NMR). Obviously, the data require three sets of Fourier transforms. 3-D data sets require a long time to acquire, can be challenging to process and interpret, and take up a lot of computer space. Nevertheless, such experiments are now routine in protein structural analysis.

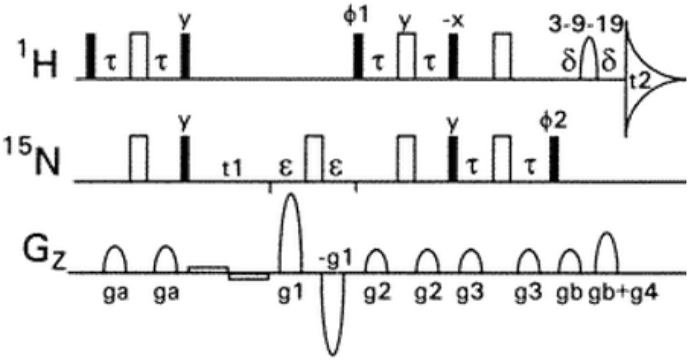
Below are examples of 2-D pulse programs, one for HSQC and one for TROSY. Note that there are many variations of these basic 2-D experiments, which all yield a similar basic spectrum, but employ different phase cycling/gradient schemes, types of water suppression, pulse sequence tricks to optimize sensitivity or filter out artifacts, etc. Note that in both cases the incremental delay (evolution) period of the pulse program is indicated by t_1. Both of these sequences involve the use of z-axis pulsed field gradients (PFG and G_z) and involve sophisticated water peak suppression methods. You can see that in the HSQC spectrum ^{15}N is decoupled from the detected protons during acquisition of the FID, not so in the TROSY sequence.

HSQC (Mulder, Otten, Scheek. J Biomol NMR. 2011 51:199-207)



Explanation from the above paper: SUMMARY: Figure shows the PFG-SE 15N-1H HSQC pulse sequence of Kay et al. (1992), in its water flip-back implementation (Zhang et al. 1994), as used in this study. The 'encoding' and 'decoding' gradients, G4 and G7, respectively, ensure proper 'winding' and successive 'unwinding' of the nuclear spin magnetizations to a degree proportional to coherence order and position in the sample. Following the recipe described in the original paper (Kay et al. 1992), two scans of opposite phase-modulated data are recorded, and manipulated prior to Fourier transformation, to generate a 2D spectrum with absorption line shapes in both frequency dimensions. DETAILED CAPTION: This is the pulse sequence of the 2D PFG-SE 1H-15N HSQC experiment used in this paper. Narrow and wide filled bars indicate 90° and 180° RF pulses, respectively, applied along the x axis, unless otherwise indicated. The 1H carrier is centered at the water resonance (4.76 ppm) and proton pulses are applied with a field strength of $\omega 1/2\pi$ = 37.3 kHz. Proton decoupling is achieved using GARP-1 decoupling with $\omega 1/2\pi$ = 1.25 kHz. The 90° water flip-back pulse after the first INEPT in the sequence (open dome) has a rectangular shape and a length of 2 ms. The 15N carrier is centered at 119 ppm, and nitrogen pulses were applied with a field strength $\omega 1/2\pi$ = 4.7 kHz. Delays are: Δ = 2.3 ms, δ = 1.5 ms, and ε = 0.2 ms. Phase cycling is: $\phi 1$ = {x,-x}, $\phi 2$ = x, and ϕrec = {x,−x}. The gradient strengths (G/cm) and durations (ms) are: g0 = 0.9 (t1/2), g1 = 5.3 (1.0), g2 = 14.1 (0.5), g3 = 22.1 (0.5), g4 = 26.6 (1.25), g5 = variable (0.15), g6 = variable (0.15), and g7 = 52.3 (0.125). Two data sets are recorded (in an interleaved manner) with G7 inverted for each data set together with inversion of $\phi 2$. The two data sets are manipulated in order to generate States type hypercomplex data (see text). Axial peaks are moved to the side of the spectrum by concomitant inversion of $\phi 1$ with the receiver phase for every other t1 increment

TROSY (J. Weigelt *J. Am. Chem. Soc.,* 1998, 120, 10778–10779)



Explanation from caption of above JACS paper: Pulse scheme with spin-state selective-, sensitivity-, and gradient-enhanced 15N−1H magnetization transfer. Filled and open pulses are applied with flip angles of 90° and 180°, respectively. Bell-shaped pulses are selective. Pulse phases are x unless indicated otherwise. P- and N-type signals of the most slowly relaxing cross-peak component is selected with φ1/φ2 = y/x and PFG signs as indicated (P-peak), and with φ1/φ2 = −y/−x and inverted sign of the PFGs g1 (N-peak). PFGs are applied with a duration of 1 ms and a sine-bell-shaped envelope. (a) 15N SG-TROSY. Parameters: ε = δ = 1.4 ms (PFG duration + recovery delay), τ = 1/(41JNH) = 2.7 ms, g1,2,3,4,a,b = 15, 1.5, 1.5, 3.05, 1.5, 3.0 G/cm, bipolar gradients16 0.5 G/cm. The phase of the first 90° (15N) pulse may be phase-alternated together with the receiver phase. Axial peak artifacts are shifted to the side of the spectrum by inverting the phases of all 15N pulses before t1 and the receiver phase with each t1 increment. The pulse sequence provides water flip-back. For enhanced water suppression, the last 180° (1H) pulse is implemented as a 3−9−19 pulse.15 With magic angle PFGs,17 acceptable water suppression was also obtained with a hard 180° pulse as the last 1H pulse. This requires, in addition, phase inversion of the last two 90° (1H) pulses to maintain the water flip-back effect.

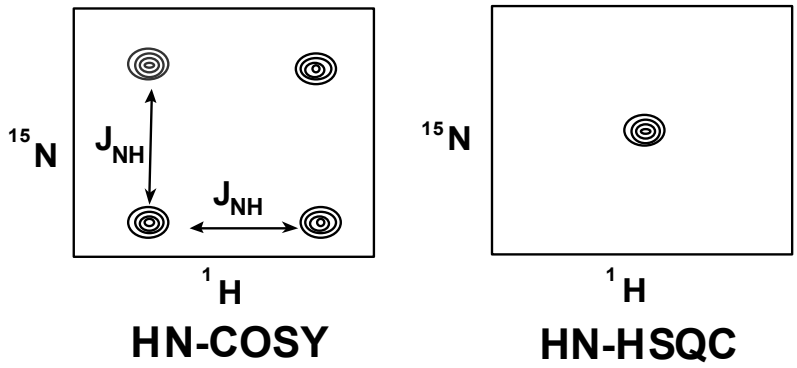
The 2-D ^{1}H,^{15}N-HSQC and TROSY Experiments

Of special importance for protein NMR are the HSQC and TROSY heteronuclear correlation experiments, which yield spectra of very similar appearance (but not completely identical). These experiments have a direct ^{1}H dimension (x-axis) and an indirect X nucleus (usually ^{15}N) dimension (y-axis), and give crosspeaks that connect protons to directly bonded (and therefore J-coupled) ^{15}N sites.

HSQC and TROSY spectra are of importance because:

- These spectra are routinely used to assess whether a protein has ordered 3-D structure or not.
- These spectra are routinely used to assess the feasibility of tackling a protein's structure using NMR methods. (Can most peaks be resolved? Are most or all expected peaks seen?)
- These spectra are often used as reporter spectra to monitor titrations involving protein-ligand, protein-protein, or protein-nucleic acid interactions. Used to monitor peaks shift or the appearance of a new set of peaks appear as the titrate proceeds). Each amide has its specific crosspeak in the spectrum that can be monitored individually.
- These experiments are conveniently combined with relaxation measurement experiments to determine ^{15}N T_1 and T_2, as well as the ^{1}H-^{15}N heteronuclear NOEs (used to assess motion).
- These experiments are used as the foundation of almost all 3-D and 4-D NMR experiments used to study proteins (NOESY-HSQC, NOESY-TROSY, HNCA, HNCO, HNCACB etc.)

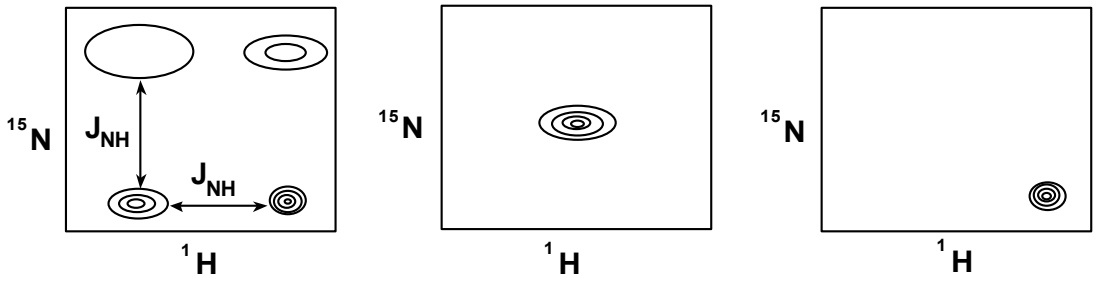
There are significant differences in the TROSY and HSQC pulse sequences that are exploited to focus on slightly different aspects of the ^{1}H-^{15}N correlation spectrum. Consider a very old fashioned 2-D heteronuclear ("HNCOSY") correlation spectrum for a single proton attached to a single nitrogen, with no decoupling in either dimension. For a small molecule, you would get a 2-D quartet of peaks, all with the same linewidths.



HN-COSY **HN-HSQC**

HSQC is used for situations like this. In the HSQC spectrum, the J-coupling in both direct and indirect dimension is effectively removed (decoupled). As a consequence, each ^{1}H-^{15}N pair gives only a single contour peak, which is located right at the center where the 4 peaks for the non-decoupled situation would be. HSQC works just fine for well-folded proteins up to about 20 kDa. Note however, that some amide ^{1}H-^{15}N sites may not yield peaks. This is for the case where the peaks are very broad, most often due to intermediate motions on the NMR time scale that can severely broaden the peaks. Peaks can disappear either because of the cancellation of "antiphase" magnetization that can occur during the HSQC pulse sequence for very broad peaks and/or because the peak has such low intensity (because of its breadth) that it cannot be seen above the noise.

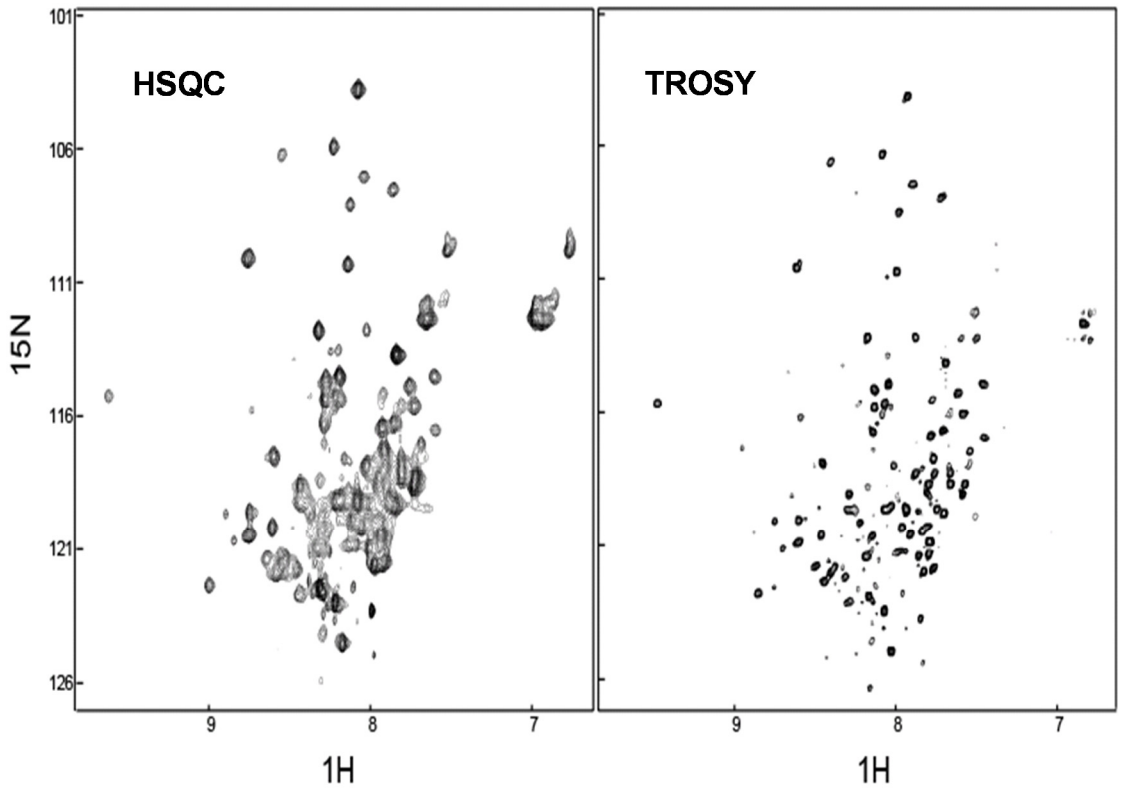
Consider now the case where one is working at NMR fields of >500 MHz and with proteins >20 kDa. The fully non-decoupled heteronuclear correlation spectrum for our single ^{1}H-^{15}N pair would appear something like what appears below-left.



HN-COSY **Classical HN-HSQC** **HN-TROSY**

The four peaks are seen to now have different linewidths. One peak is very broad (Anti-TROSY), one peak is very sharp (TROSY), and the other two are intermediate (Semi-TROSY). This is due to the "TROSY effect", which is seen for amide ^{1}H-^{15}N resonances of large molecules and complexes at high magnetic field. If you now imagine running HSCQ on this sample (middle), you would get a single average peak that has a linewidth that is the average between all four components of the quartet (in some cases, the peaks may be missing completely because of antiphase cancellation). The 2-D TROSY spectrum will differ from the HSQC spectrum. Instead of decoupling in both dimensions to collapse all four components of the quarter to an average single peak (singlet), spin gymnastics is imposed in the TROSY experiment to select only the single sharp component of the quartet for detection (the lower right TROSY peak), with the other 3 components being filtered out. TROSY therefore leads to a spectrum with sharper peaks (better resolution) for very large molecules and complexes than HSQC. Indeed, for reasons not detailed here, amide ^{1}H-^{15}N peaks are often seen in TROSY spectra but are invisible in HSQC spectra (see explanation in Sanders and Soennichsen, *Mag Res Chem*, 44: S24–S40, 2006). Since even small membrane proteins are effectively much larger once solubilized in micelles, bicelles, or other model membranes, TROSY spectra of membrane proteins are usually of higher quality then HSQC spectra. Note, however, that for smaller molecules, HSQC is preferred because you "save" all four components of the J quartet and combine them to one signal, leading to better signal-to-noise than for TROSY. In TROSY some of the magnetization is thrown out to focus on the single component of the quartet

(lower signal-to-noise relative to HSQC for smaller molecules). Shown below is a comparison of the TROSY and HSQC spectra of the same protein/micelle complex in the same sample.



You should be aware that there are many versions of both the 2-D HSQC and 2-D TROSY experiments. These individual versions vary based on:

(1) How phase cycling and gradients are used. The need for phase cycling can be reduced by sophisticated use of pulsed field gradients (PFGs). For example, in the Weigelt TROSY sequence (the one shown in detail above), there is no need to complete a phase cycle before saving the FID for an increment of the 2-D series. It is a "single scan" experiment in that the information content in every FID transient of the experiment is exactly the same. There can be some big advantages to pulse sequences using PFGs to reduce or eliminate the need for phase cycling, which include the fact that the receiver can be set to an optimal setting after one scan, since all filtering out of unwanted signals (from detergent, for example) occurs before generation of the FID, not by adding and subtracting FIDs at the end of a phase cycle.

(2) The type of water suppression used. Most modern pulse sequences use sophisticated methods to suppress the huge water peak so a decent receiver gain can be set (because the water peak no longer dominates the FID). It is also used to make sure the residual water peak does not bleed into the 2-D spectrum as an artifact. One of the goals is to suppress the water peak, but avoid pre-saturating it, which might wipe out the peaks from amide sites undergoing H-H exchange with water.

(3) How water and other peaks (such as detergent peaks) that involve protons NOT coupled to ^{15}N are filtered out (removed). Some sequences are more efficient at this than others, particularly for cases where it is not just the water peak that has to go, but also (sometimes huge) peaks from detergent or other high-concentration molecules present in your sample (buffer, EDTA, etc.). The very best sequences are extraordinarily efficient at removing unwanted peaks.

(4) How S/N is optimized. Some pulse sequences involve tricks to enhance the signal-to-noise. For example, in a bare bones TROSY spectrum the signal from 3 out of 4 components of each 2-D ^{1}H-^{15}N quartet is discarded, leaving only the signal from the single sharp component for detection. However, in some "sensitivity-enhanced" version of the pulse sequence, some of the magnetization from the 3 broad components of each quartet are transferred before detection of the sharp component, thereby enhancing

the S/N for the peak that is actually detected.

(5) How long the sequence is. Adding "bells and whistles" to pulse programs often means adding extra pulses and delays, meaning that the time it takes to run the pulse sequence is increased. This can be a problem. If the T_2 values for the amide ^{1}H-^{15}N peaks are on the same order of time or less than it takes to run the pulse sequence then you will actually lose most or all signal to relaxation before you finally turn on the detector and collect the FID. (!) For large proteins and complexes where the T_2 values can be short (often only 10-20 msec for immobile backbone 15N sites in molecules and complexes in the 100 kDa range), this can be a big problem. What this means is that sometimes the "fancy" version of a TROSY sequence (such as the "sensitivity-enhanced" version) is NOT a good choice because of the extra time that extra pulses and delays add to the pulse sequence.

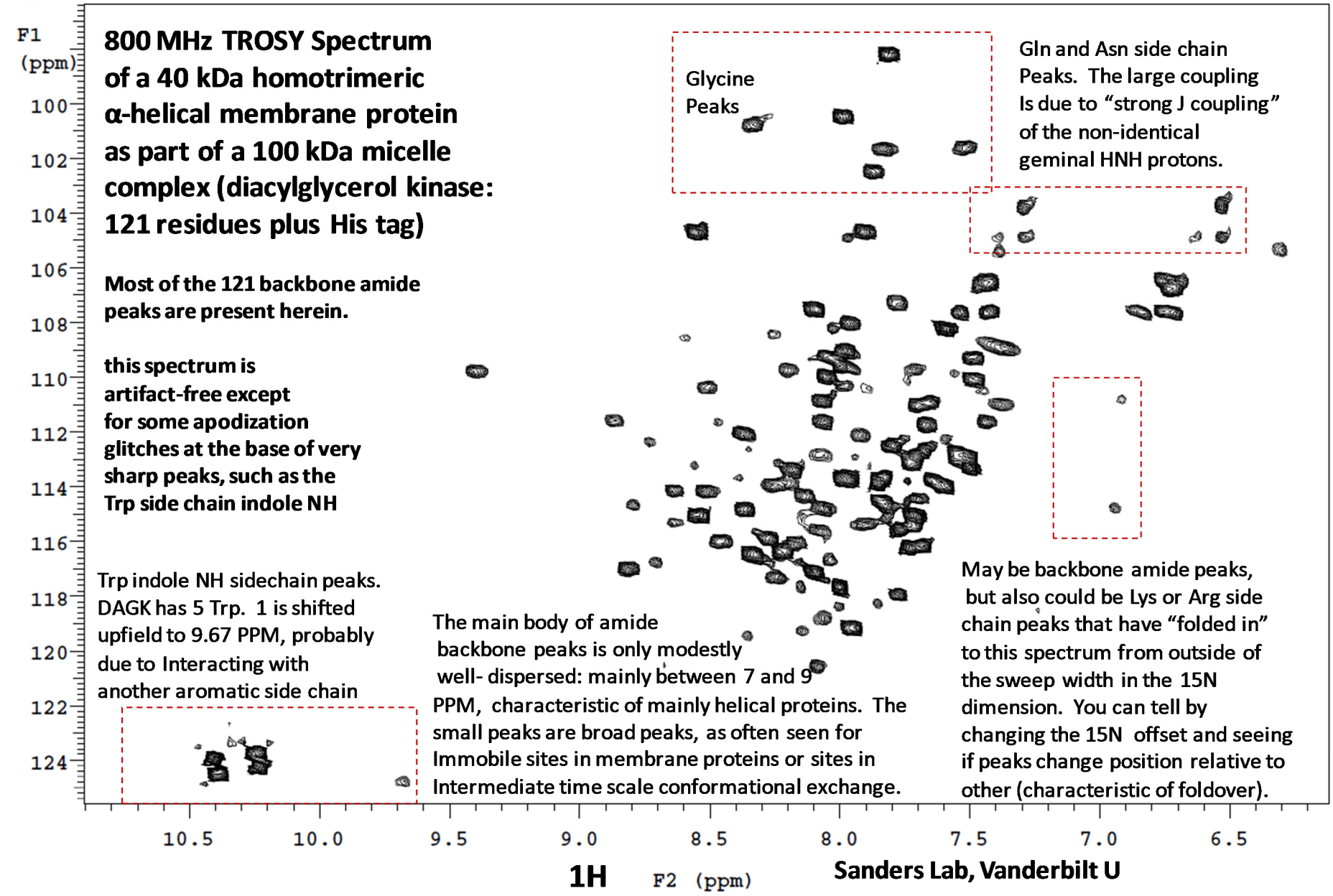
So, when you choose which version of HSQC or TROSY to run, make sure it is the RIGHT version. At least for work in the Sanders lab involving micellar or bicellar membrane proteins, there have been many instances where the wrong version of TROSY was run, leading to sub-optimal spectra. I repeat: *always verify that you are running the correct version.*

A Word on Sample Prep for HSQC and TROSY

Because HSQC and TROSY are using run in ^{1}H,^{15}N-mode the focus of these experiments is on amide protons. Amide protons will, of course, exchange with protons from water. For this reason, HSQC and TROSY samples normally contain only just enough D2O for purposes of field-frequency locking (5%-10%), otherwise the amide protons would all be replaced with deuterons.

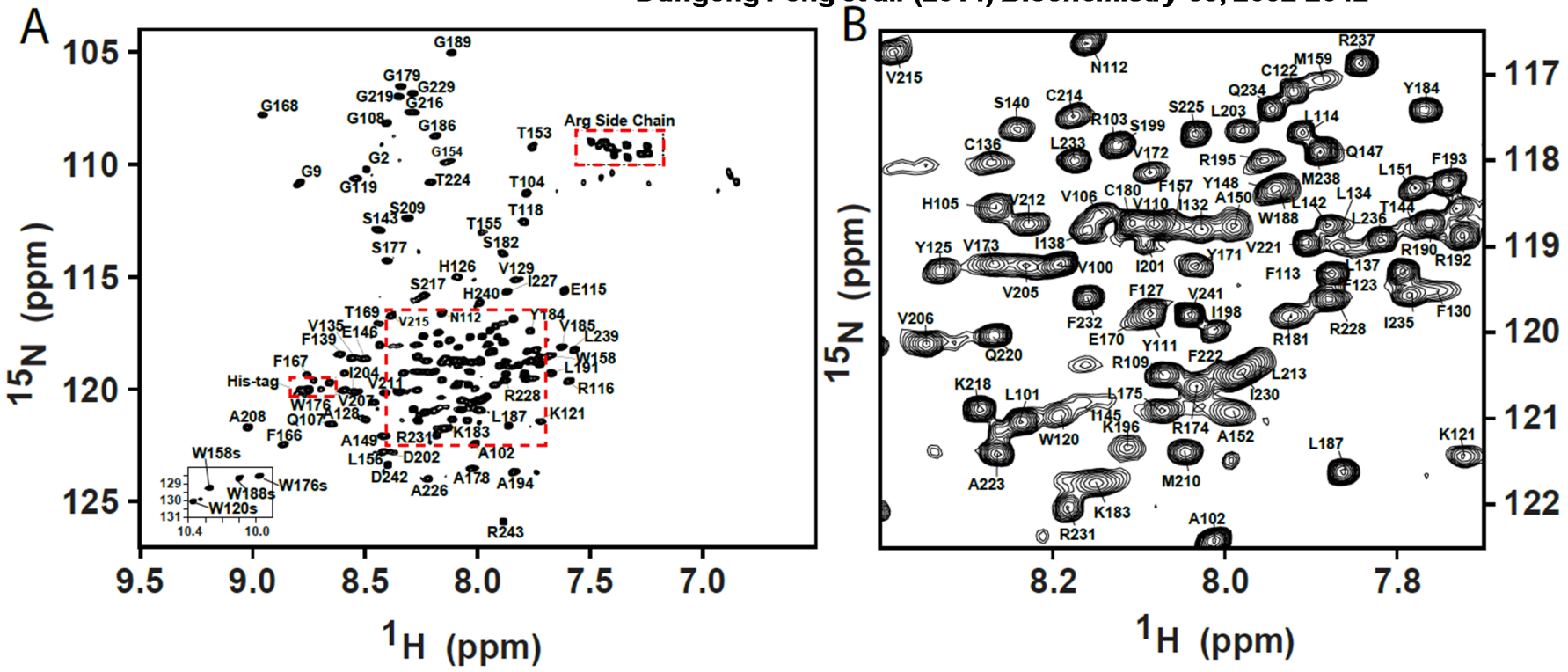
You also should recognize that at neutral and slightly basic pH, amide exchange rates between amide protons and water protons can become significant. This can sometimes cause 1H-15N peak disappearance due to intermediate or fast exchange between dilute amide proteins and ca. 50 molar water, especially at higher temperatures. In the Sanders lab we tend to stick with samples that are pH 6.5 or below to avoid this problem.

Annotated Gallery of (Mostly) TROSY or HSQC Spectra Follows…

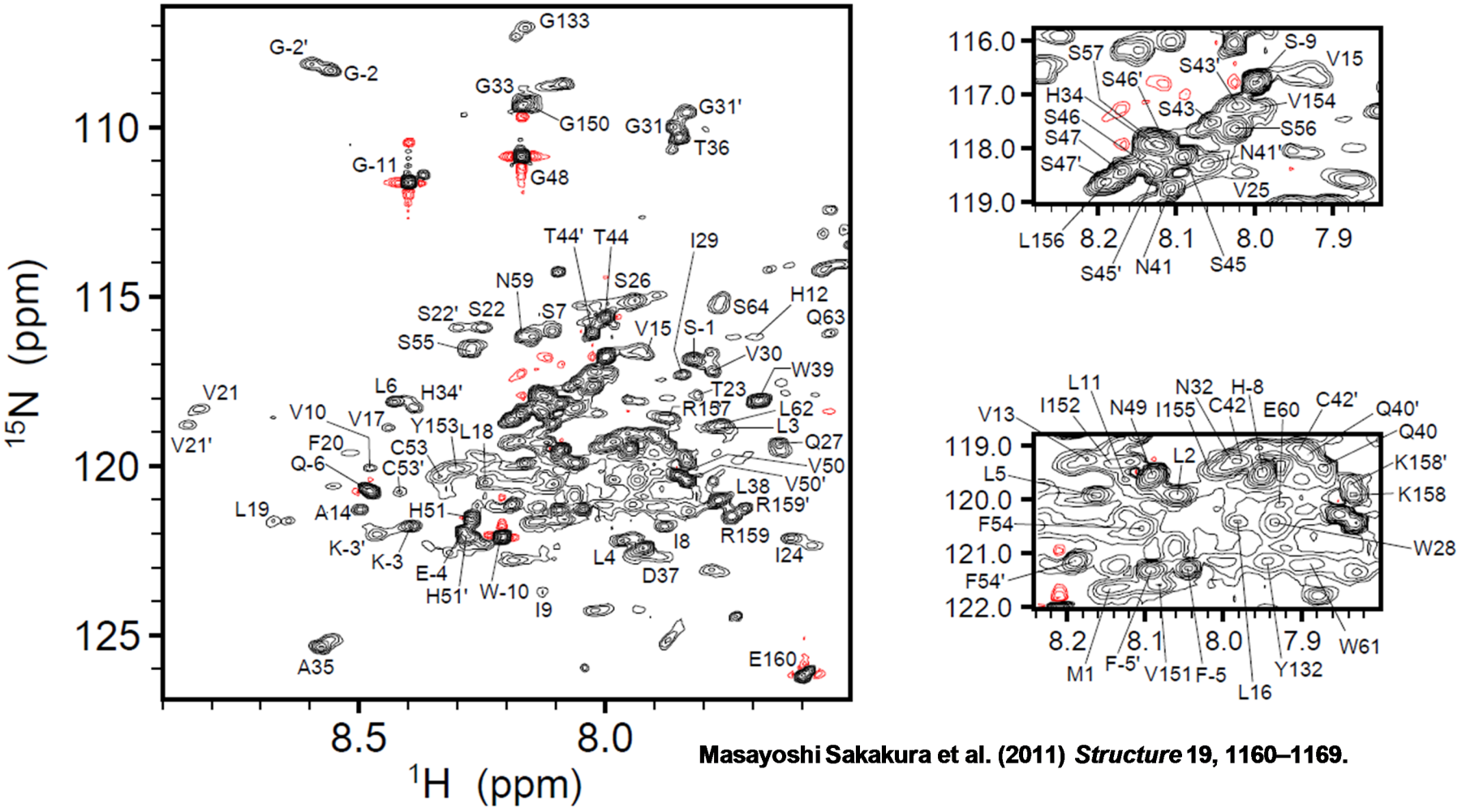

15N
F1 (ppm)
800 MHz TROSY Spectrum of a 40 kDa homotrimeric α-helical membrane protein as part of a 100 kDa micelle complex (diacylglycerol kinase: 121 residues plus His tag)
Most of the 121 backbone amide peaks are present herein.
this spectrum is artifact-free except for some apodization glitches at the base of very sharp peaks, such as the Trp side chain indole NH
Glycine Peaks
Gln and Asn side chain Peaks. The large coupling Is due to "strong J coupling" of the non-identical geminal HNH protons.
Trp indole NH sidechain peaks. DAGK has 5 Trp. 1 is shifted upfield to 9.67 PPM, probably due to Interacting with another aromatic side chain
The main body of amide backbone peaks is only modestly well- dispersed: mainly between 7 and 9 PPM, characteristic of mainly helical proteins. The small peaks are broad peaks, as often seen for Immobile sites in membrane proteins or sites in Intermediate time scale conformational exchange.
May be backbone amide peaks, but also could be Lys or Arg side chain peaks that have "folded in" to this spectrum from outside of the sweep width in the 15N dimension. You can tell by changing the 15N offset and seeing if peaks change position relative to other (characteristic of foldover).
100
102
104
106
108
110
112
114
116
118
120
122
124
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
1H F2 (ppm)
Sanders Lab, Vanderbilt U

BELOW Assigned 900 MHz TROSY spectrum of the 143 residue voltage sensor domain of the human KCNQ1 potassium channel in detergent micelles. This protein has 4 transmembrane segments. The inset to the lower left of the panel shows the Trp side chain indole resonances.

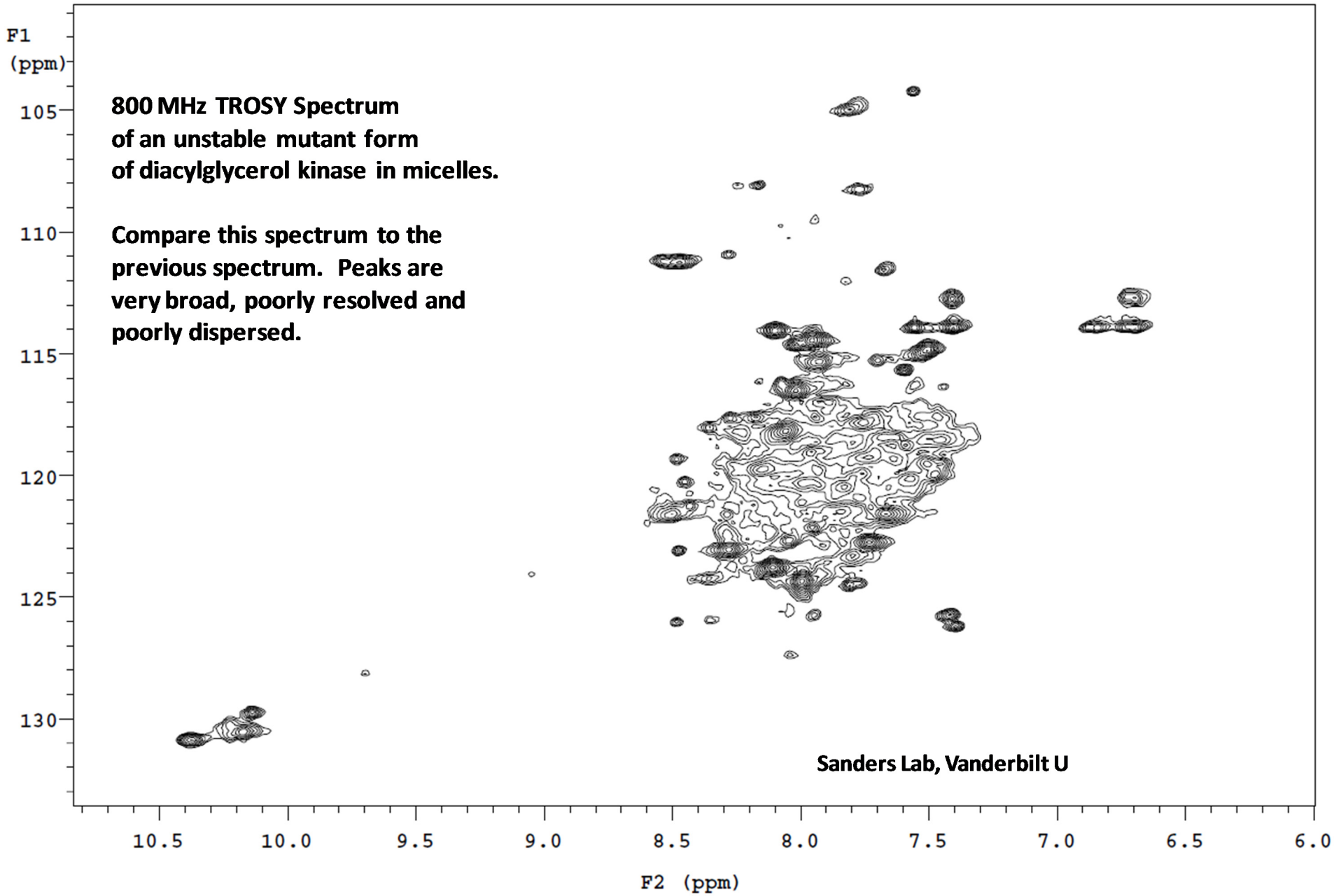
Dungeng Peng et al. (2014) *Biochemistry* 53, 2032-2042

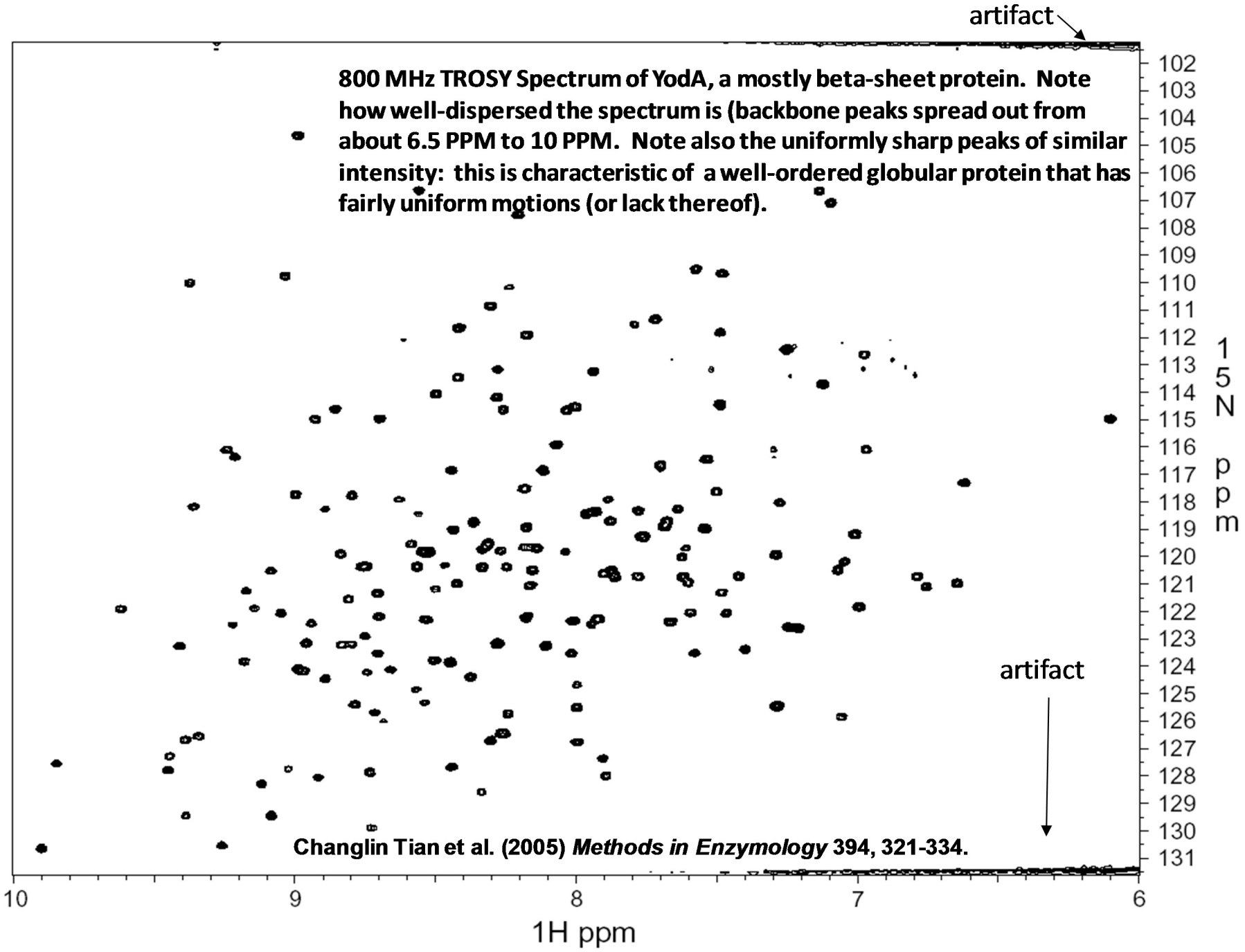


BELOW NMR Spectrum of a partially unfolded helical membrane protein, peripheral myelin protein 22, a tetraspan protein, in micelles. Only a fraction of the 160 expected backbone amide peaks are seen and only a fraction of those could be assigned. Under the conditions of this sample three of the protein's transmembrane segments form a molten globular bundle, while the 1st transmembrane segment is dissociated from that bundle. Peak linewidth varies dramatically, illustrating that a wide range of motions and associated rates is present within this protein.



Masayoshi Sakakura et al. (2011) *Structure* 19, 1160–1169.


F1
(ppm)
105
110
115
120
125
130
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
F2 (ppm)
800 MHz TROSY Spectrum
of an unstable mutant form
of diacylglycerol kinase in micelles.
Compare this spectrum to the
previous spectrum. Peaks are
very broad, poorly resolved and
poorly dispersed.
Sanders Lab, Vanderbilt U


artifact
800 MHz TROSY Spectrum of YodA, a mostly beta-sheet protein. Note how well-dispersed the spectrum is (backbone peaks spread out from about 6.5 PPM to 10 PPM. Note also the uniformly sharp peaks of similar intensity: this is characteristic of a well-ordered globular protein that has fairly uniform motions (or lack thereof).
artifact
Changlin Tian et al. (2005) Methods in Enzymology 394, 321-334.
102
103
104
105
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107
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110
111
112
113
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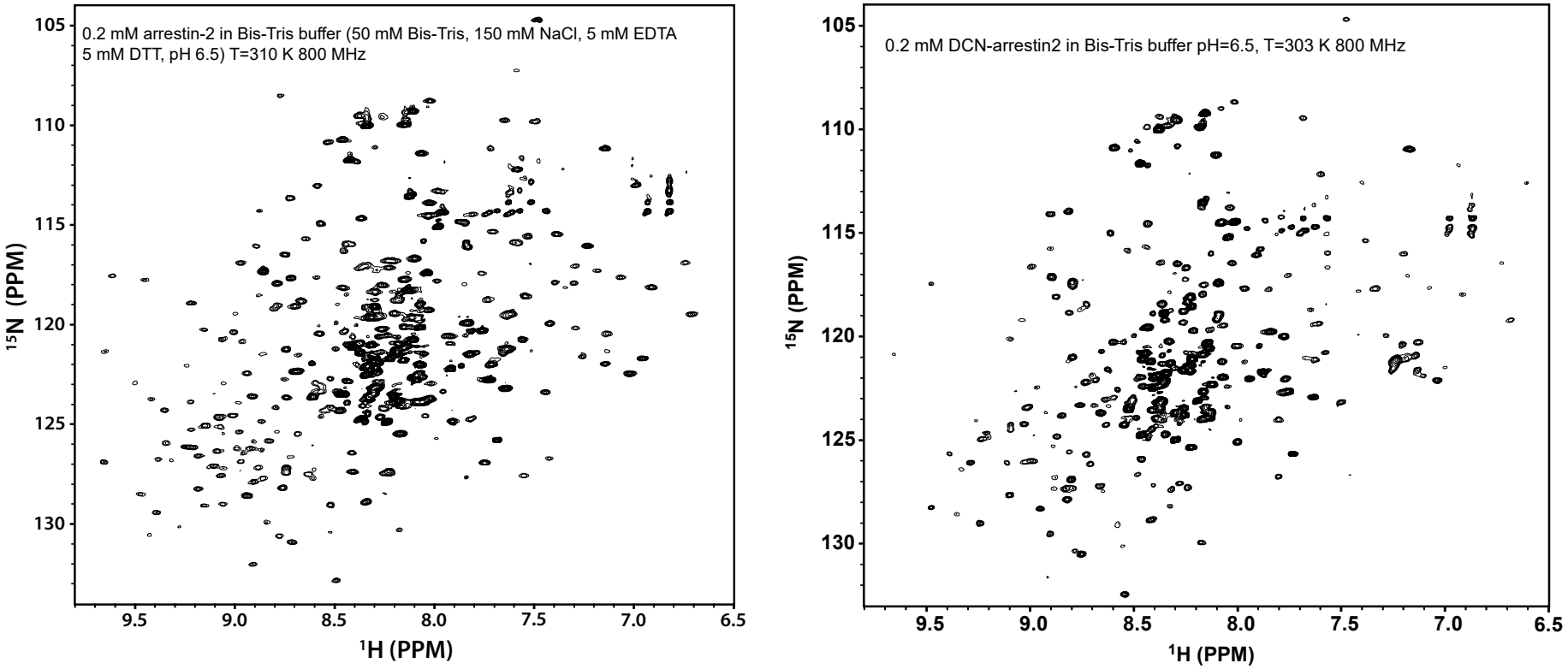
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
15N ppm
10
9
8
7
6
1H ppm

BELOW Comparison of 800 MHz TROSY sprectra of human arrestin-2 (300 residues) acquired for non-deuterated protein (left) versus perdeuterated protein (right). There may be a modest enhancement in resolution for the deuterated sample, but this result is typical of 2-D TROSY: they are usually not very sensitive to sample perdeuteration. Where perdeuteration really matters is in 3-D experiments with a 13C dimension involving 13C with directly-bonded protons: in this case perdeuteration is often needed to see peaks.

Upwards of 200 amide peaks are seen in the spectra below (of 300 expected). It was possible to assign about 150 peaks (not shown). As you might guess from these spectra, arrestins are mainly beta sheet proteins, but also contain some helices and disordered segments.



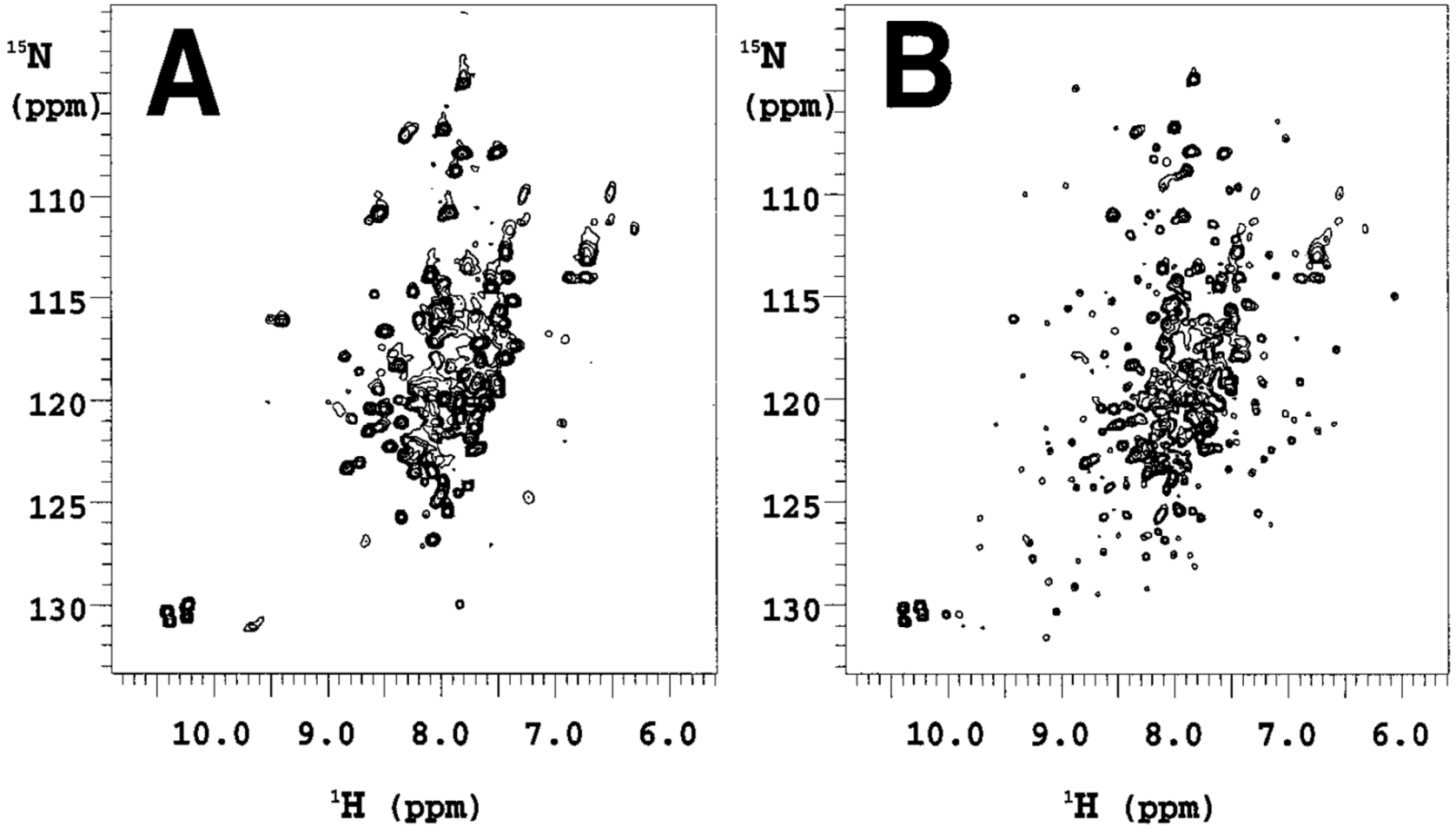
Background work for: Tiandi Zhuang, Qiuyan Chen et al (2013) *PNAS* 110, 942-947.

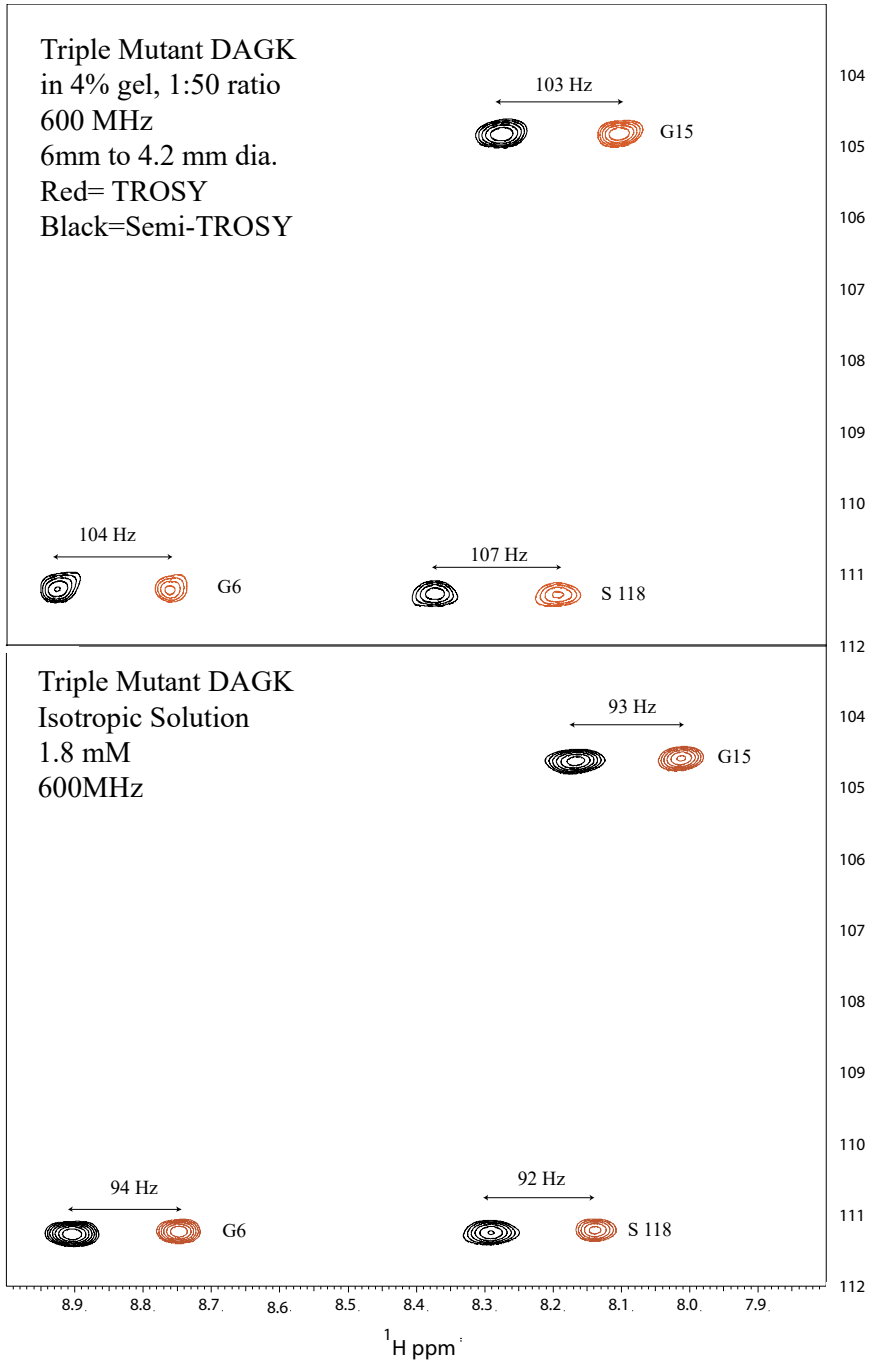
600 MHz TROSY Spectra of a Pure Protein and an Impure Protein.

A. Spectrum of pure diacylglycerol kinase in micelles. Note that the spectral quality is not as high as the previously-shown 800 MHz spectrum. This is because (1) the TROSY effect is nearly optimal at 800 MHz (sharper peaks), but not at 600 MHz (broader peaks) and (2) spectra dispersion is lower at 600 MHz (more peaks per Hz) than at 800 MHz.

B. Same as A except the DAGK sample is contaminated with a second protein, YodA. Note that the YodA peaks are sharp even when the TROSY effect is not optimized because it is a relatively small water soluble protein.

Oxenoid, K. et al. *Biochemistry 40,* 5111-5118 (2001).





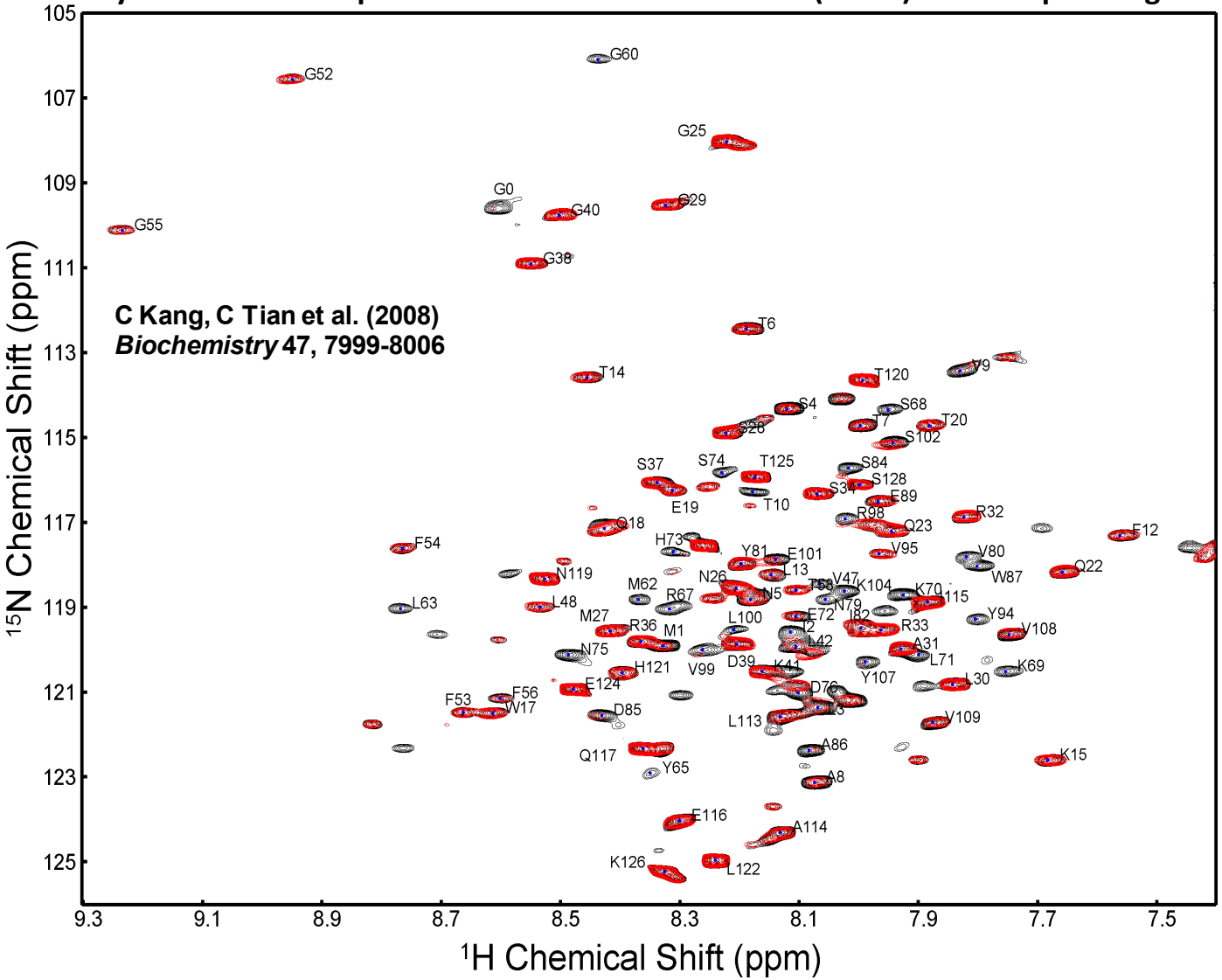
Example of use of TROSY to measure the values for residual dipolar couplings for 3 peaks selected from TROSY spectra of a gel-aligned DAGK sample (top spectrum) versus an isotropic sample (bottom).

In each case a pair of TROSY spectra were acquired for each sample. The red spectrum is the traditional TROSY spectrum where the selected component of The 1H-15N quartet is the sharpest (lower right) Component. To acquire the black spectrum, the TROSY pulse sequence was altered so that the Second sharpest component of the quartet is observed (the lower left component. The 1H-15N couplings seen in the isotropic sample are J-only, whereas the couplings seen from the aligned sample are J+D.

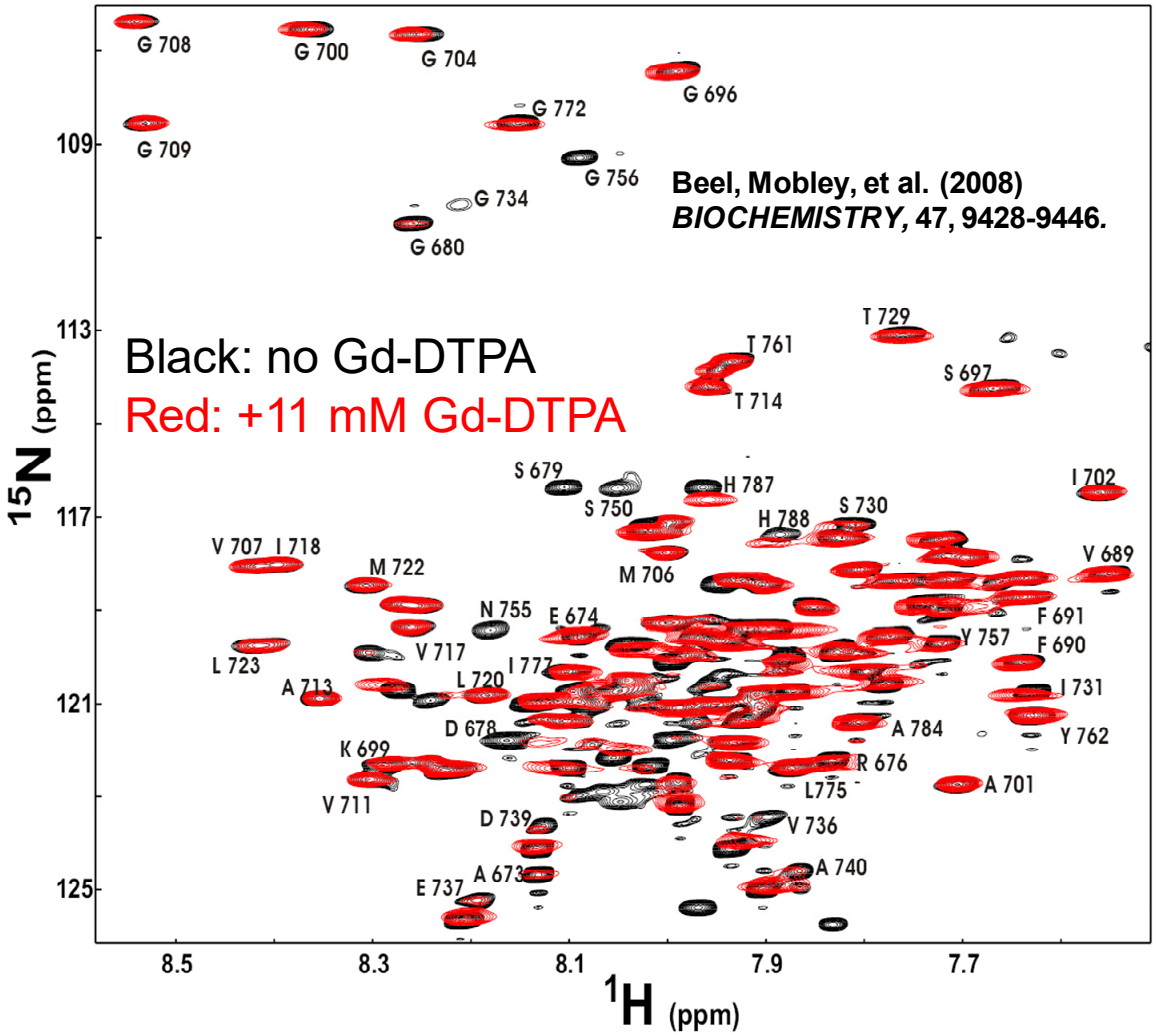
Note, these couplings are often measured in the 15N dimension. To measure them as we have in the 1H dimension, perdeuterated protein needs to be used to avoid complications due to J coupling between the Cα proton and both the amide 15N and 1H. It is also probably prudent to avoid using 13C labeling as well.

Van Horn WD, Kim HJ et al. (2009) *Science* 324, 1726-1729.

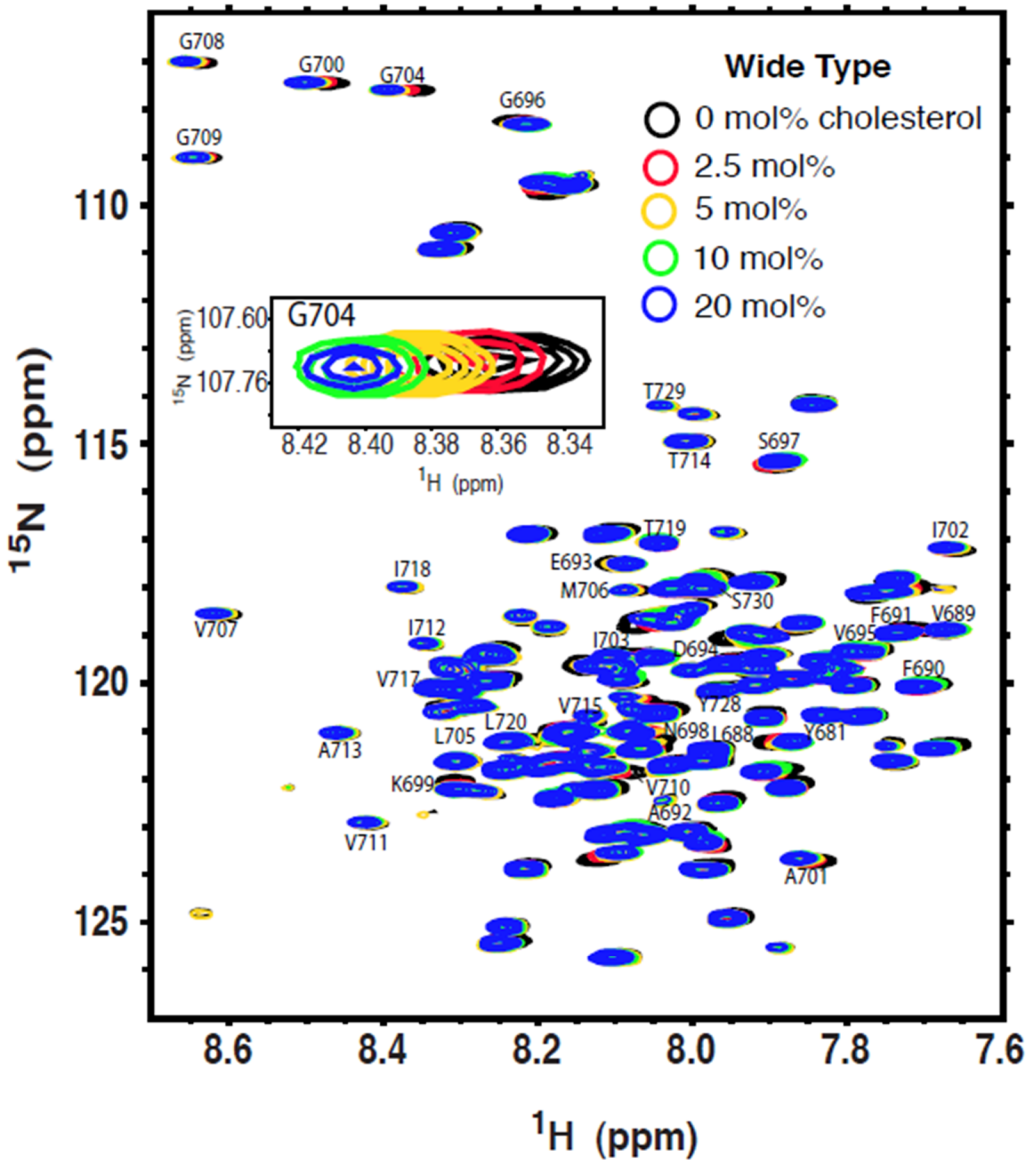
BELOW Superimposed pair of 800 MHz TROSY spectra used to quantitate paramagnetic relaxation enhancement (PRE) for a nitroxide spin-labeled protein. Probe-amide proton distances are derived from each PRE. The protein is KCNE1, a single span membrane protein. The site of covalent spin-labeling is S64C (in otherwise Cysless protein). The red spectrum is with the nitroxide spin label in its paramagnetic form. The underlying black spectrum is following quenching of the paramagnet. Peaks for which only the black peak is seen are from sites that are close (< 12A) to the paramagnet. Peaks where the red component is the same in intensity as the black component are from sites that are far (> 22A) from the paramagnet.

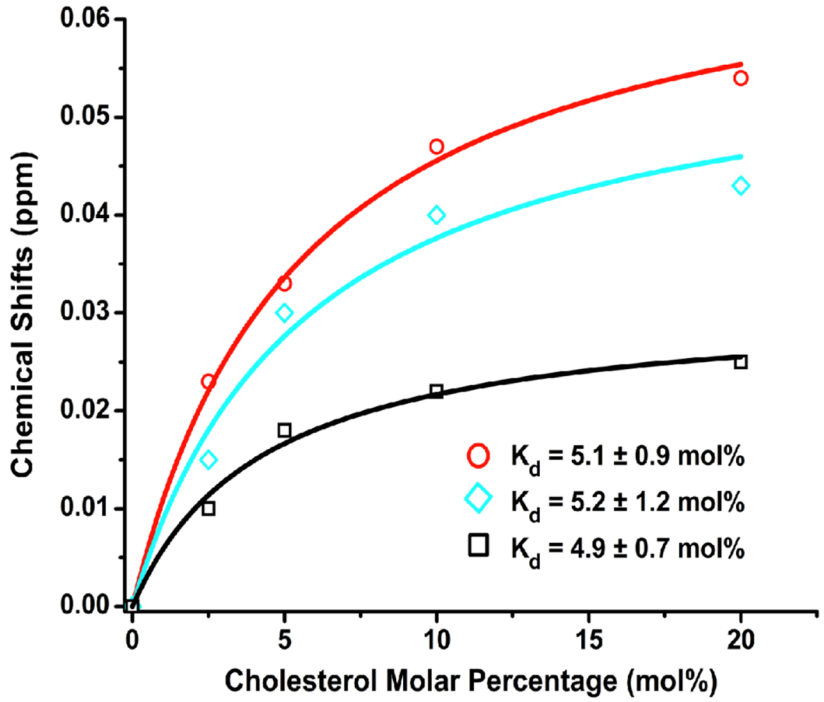


BELOW Use of 800 MHz TROSY spectra to probe the topology of a membrane protein. Here the TROSY spectra of C99 (C-terminal domain of the amyloid precursor protein, a single span membrane protein) in detergent micelles are compared in the absence (black) and presence (red) of a water soluble paramagnetic probe: the chelate complex of Gd(III) with DTPA. Peaks for which no red component is observed are from freely water-exposed sites. Peaks for which the read component has the same intensity as the black component are from sites that are buried in the micelle interior and are not accessible to the water soluble chelate.



BELOW Use of 800 MHz TROSY spectra to monitor titration of a protein with a ligand and determine to Kd for binding of that ligand. (LEFT) Superimposed series of TROSY spectra of C99 (C-terminal domain of the amyloid precursor protein, a single span membrane protein) in bicelles as it was titrated with varying concentrations of cholesterol. Some peaks shift (none split) in response to titration, characteristic of binding that has a rapid on/off exchange rate on the NMR time scale. For the three peaks that shifted the most in response to binding the shifts were plotted on the right and fit by the 1:1 binding model. Fitting of each of the three peaks yielded the same Kd, indicating all three peaks are reporting on the same binding event.

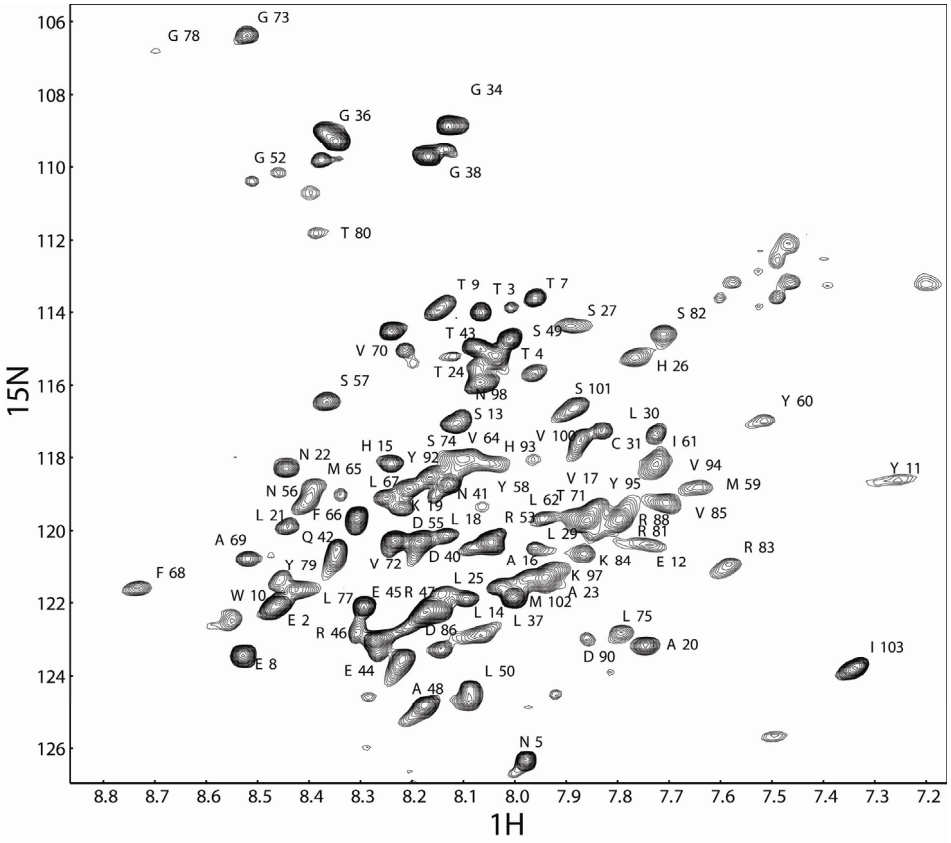




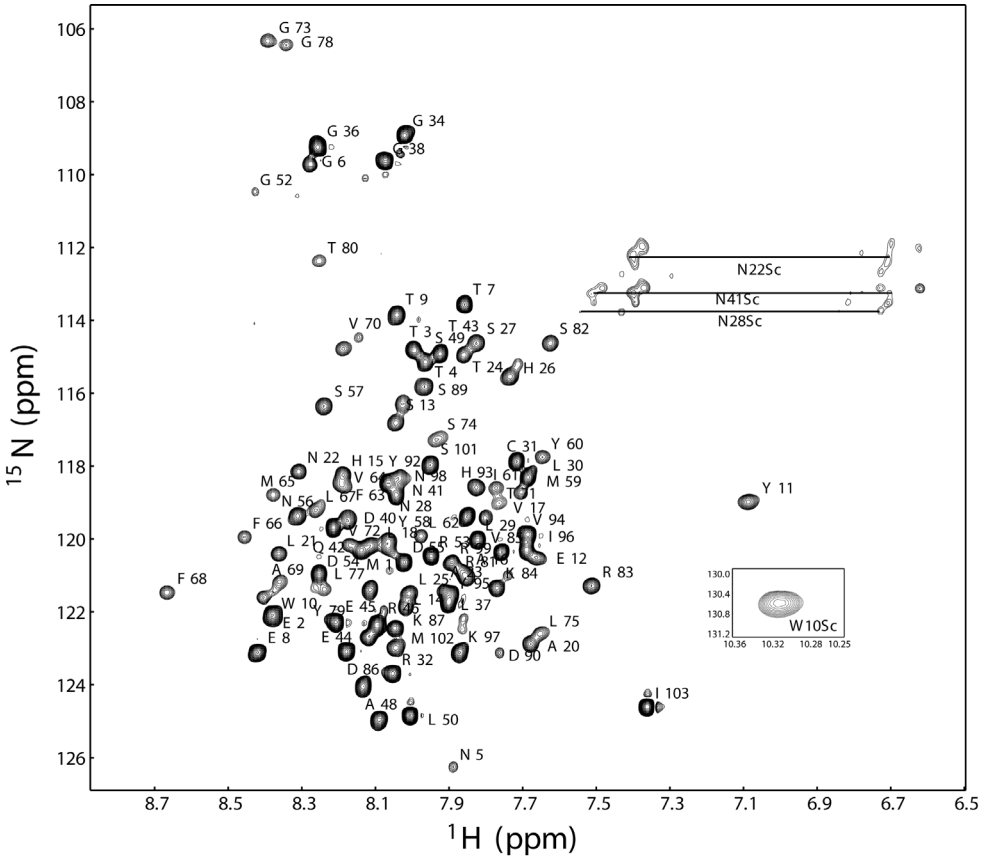
Paul J. Barrett*, Yuanli Song* et al. (2012)
***Science*, 336, 1168-1171.**

Assigned TROSY Spectra of the Same Membrane Protein (KCNE3: 1 TM span and 103 residues) in both micelles (left) and bicelles (right). In this case bicelles yielded data that was of significantly higher quality than micelles, although both spectra were assignable.

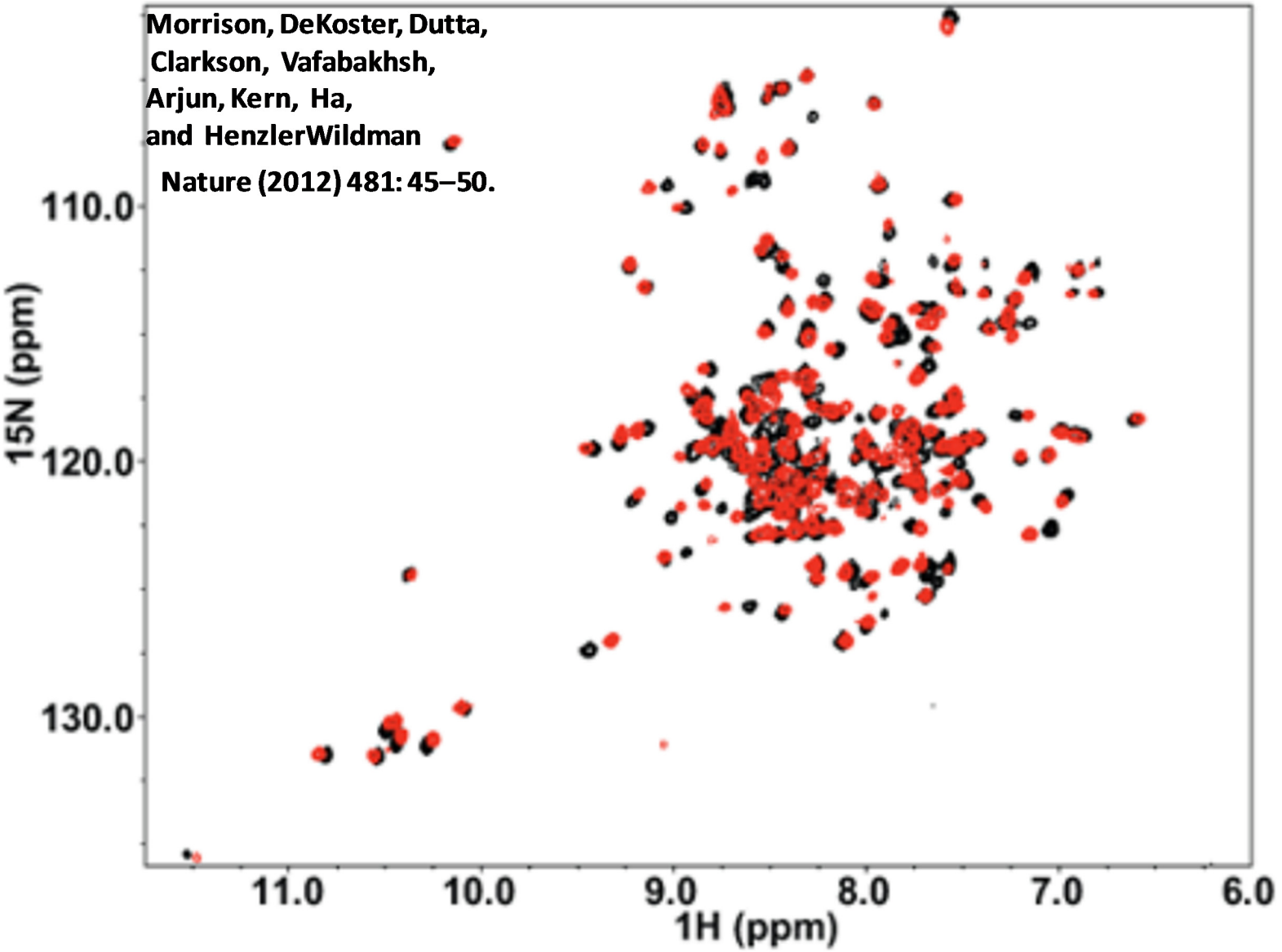
Backbone amide 1H-15N TROSY-HSQC resonance assignments for KCNE3 in LMPC micelles at pH 6.5 and 40C. The spectrum was obtained on Bruker 800 MHz.
From: Kang C, Vanoye CG, Welch RC, Van Horn WD, and Sanders CR (2010) Biochemistry 49, 653-655.



Backbone amide 1H-15N TROSY-HSQC resonance assignments for KCNE3 in DHPC-DMPC bicelles at pH 6.5 and 40C. The spectrum was obtained on Bruker 800 MHz. Note the peaks with the very large splittings in the upper right corner. These peaks are from asparagine side chain amide protons, which are "strongly coupled". From: Kang C, Vanoye CG, Welch RC, Van Horn WD, and Sanders CR (2010) Biochemistry 49, 653-655.

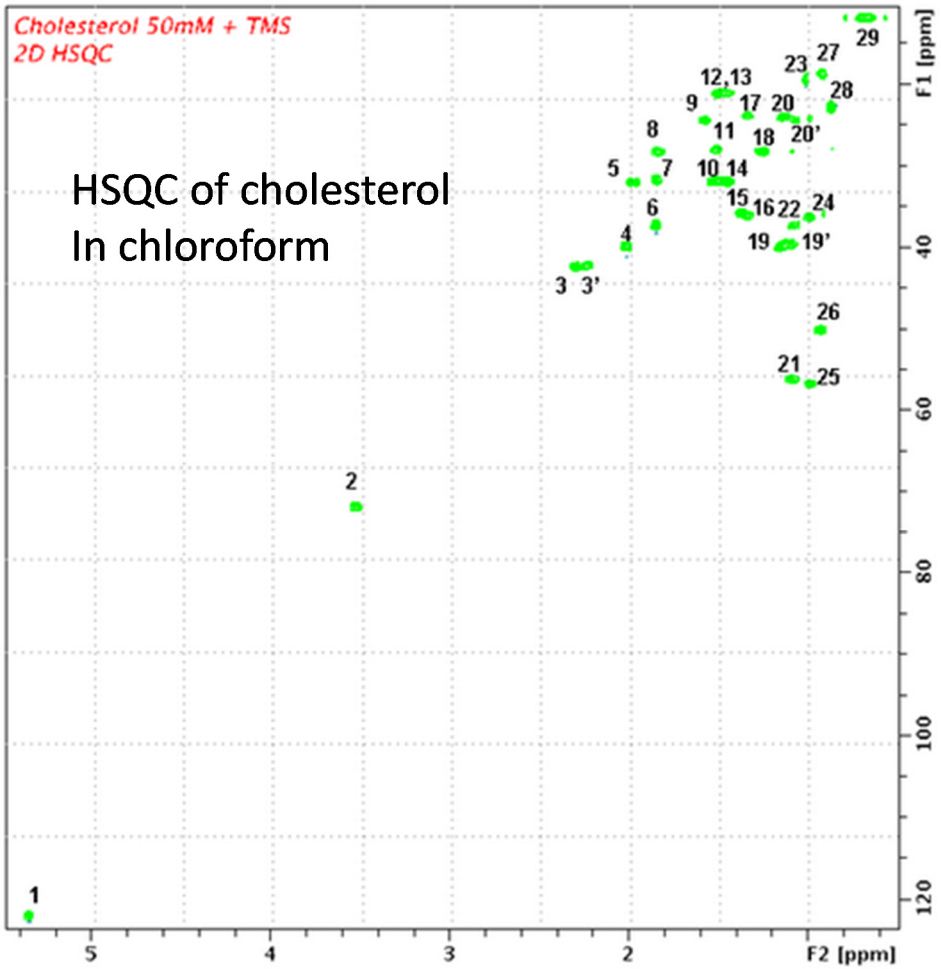


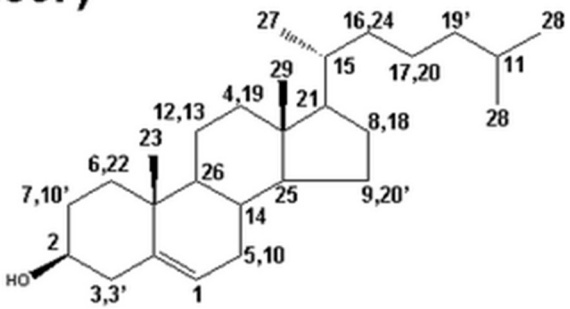
This is the TROSY spectrum of the multi-drug transporter EmrE in bicelles. This spectrum shows twice as many peaks as expected from this protein, indicating the protein adopts two conformations That are in slow exchange on the NMR time scale. The fact that the two sets of peaks have the same intensities (peak integrals) means the two conformations have the same population. This spectrum is consistent with the assymetric dimer nature of the EmrE structure. The red and black spectra are from 2 different samples with different types of bicelles.



Cholesterol (HMDB00067)

Wishart DS, Tzur D, Knox C, et al., HMDB: the Human Metabolome Database. Nucleic Acids Res. 2007





#	F2[ppm]	F1[ppm]	Intensity
1	5.3501	121.8120	6116323.97
2	3.5225	71.7482	1922587.78
3	2.2617	42.3513	1102034.34
3'	2.2295	42.1432	
4	2.0187	39.7361	1991897.09
5	1.9758	31.9632	739035.44
6	1.8414	37.2662	985801.00
7	1.8386	31.6726	1247746.63
8	1.8357	28.2584	1015036.41
9	1.5726	24.3356	1254638.03
10	1.5362	31.8834	622959.66
10'	1.5029	31.5511	
11	1.5154	27.9678	1068394.06
12	1.4954	21.0667	2046811.44
13	1.4497	21.0667	712248.41
14	1.4597	31.8695	1638557.59
15	1.3725	35.6680	1026060.38
16	1.3382	35.9586	1597388.28
17	1.3296	23.6818	1260655.91
18	1.2495	28.3310	1107382.81
19	1.1466	39.7361	1771080.56
19'	1.1176	39.5405	
20	1.1294	23.8271	787348.44
20'	1.0705	24.2188	
21	1.0837	56.2262	1391709.13
22	1.0722	37.2662	1412927.88
23	1.0065	19.5411	5916607.00
24	0.9836	36.1766	913445.72
25	0.9750	56.5894	1011071.97
26	0.9236	50.1241	1621559.28
27	0.9121	18.6694	3947413.97
28	0.8635	22.8101	7860020.00
29	0.6719	11.7683	688605.25