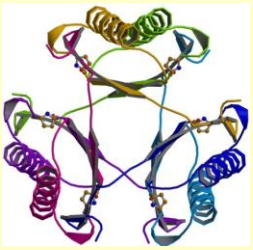
Image Formation

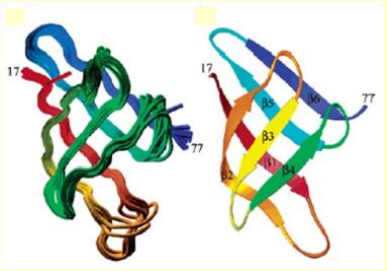
• Light Photography

λ ~ 400 - 700 nm

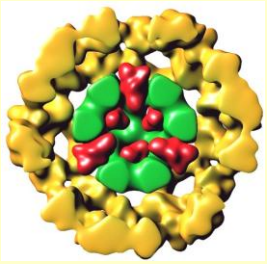


• X-Ray

λ ~ 0.1 nm



• NMR



• Electron Microscopy

λ ~ 0.001 - 0.1 nm

Structure Determination by Multidimensional NMR

Questions:

1. **NMR Basics**
   - **Spin states / Energy of transitions / What defines a "500 MHz" NMR?**
   - **Chemical shifts / How to interpret basic NMR spectra?**
2. **Many types of NMR experiments**
   - **COSY / NOESY**
3. **What are the requirements and limitations of multidimensional NMR methods?**
4. **What is the "Assignment Problem"?**
5. **How are "Assignments" made?**
6. **From peaks to secondary structure to a 3D model.**
   - **How is the protein "model" obtained?**
7. **Comparison of structure determination by X-ray vs. NMR.**

NMR Methods

Nuclear spin (nuclear spin Quantum Number *I*)

No spin: #neutrons and #protons both even - 12C, 16O
Half-integer spin (1/2, 3/2, 5/2): #neutrons + #protons odd - 1H, 13C, 15N
Integer spin (1, 2, 3): #neutrons and #protons both odd - 2H, 14N

Spin Quantum Numbers of Common Nuclei

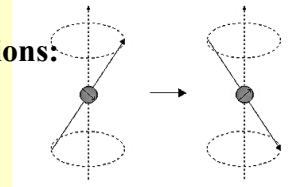
Element	^{1}H	^{2}H	^{12}C	^{13}C	^{14}N	^{16}O	^{17}O	^{19}F
Nuclear Spin Quantum No (I)	1/2	1	0	1/2	1	0	5/2	1/2
No. of Spin States	2	3	0	2	3	0	6	2

Elements with odd mass or odd atomic number have nuclear "spin".

http://www.chem.umd.edu/courses/chem243davis/

NMR Methods

Nuclear spin and the **splitting of energy levels** in a **magnetic field**



Nuclear spin quantum # "*I*" ; 2*I* + 1 orientations:

Magnetic quantum # "m; (m = -*I* to +*I*)
e.g. *I* = ½ ; *m* = -1/2 , +1/2

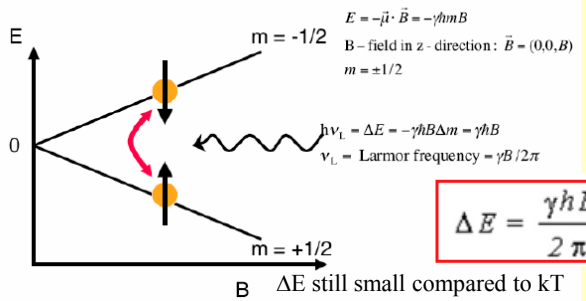
Nuclear magnetic moment

$$\mu = \frac{\gamma I h}{2\pi}$$

Energy of level "*m*"

$$E = -\frac{\gamma h}{2\pi} m B$$

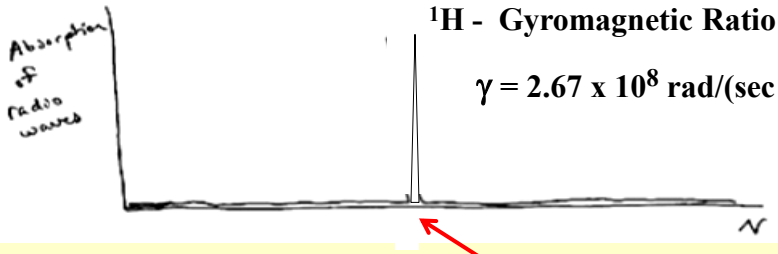
Magnetic transitions of a spin-1/2 nucleus



$$\Delta E = \frac{\gamma h B}{2\pi}$$

ΔE still small compared to kT

NMR Spectrum of Water (H_2O) in a magnetic field of 11.7 Tesla – one peak since both protons are equivalent.



^{1}H - Gyromagnetic Ratio

$\gamma = 2.67 \times 10^8$ rad/(sec Tesla)

$\Delta E = \gamma\, h\, B/2\pi = h\, \nu$ **or** $\nu = \gamma\, B/2\pi$

$\nu = [(2.67 \times 10^8 \text{ rad/(sec Tesla)})\ (11.7 \text{ Tesla})]\ /2\pi$

$\nu = 4.97 \times 10^8$ /sec = 497 MHz

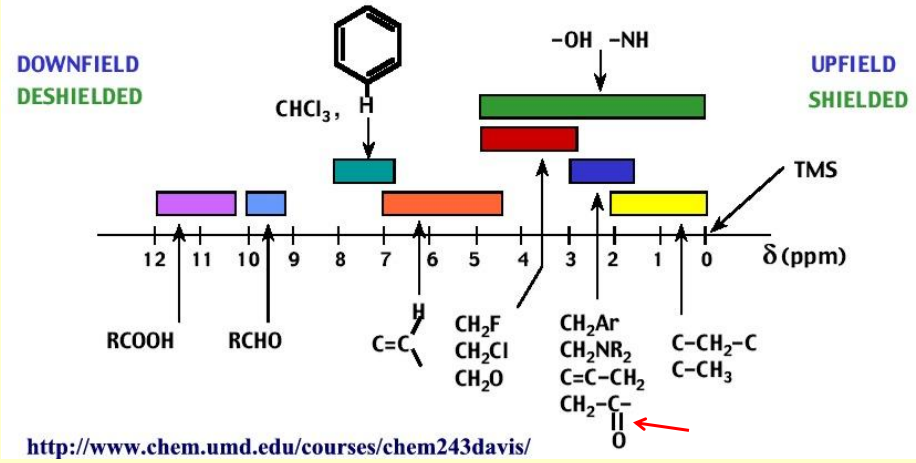
What is a Tesla? How strong is a magnetic field of 1 T?

1 T (tesla) = 10^4 gauss

1 gauss = 10^{-4} kg C^{-1} s^{-1}

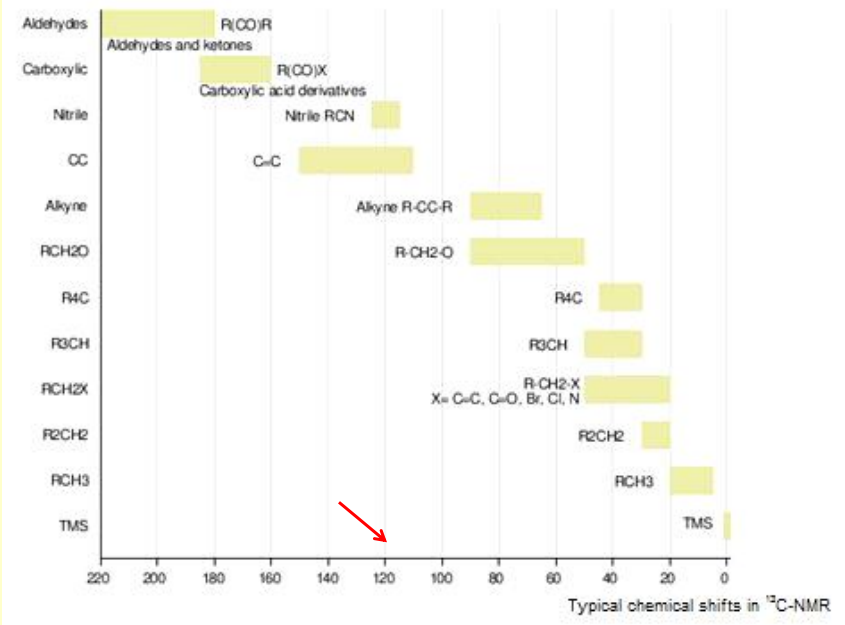
- 10^{-9}–10^{-8} **gauss**: human brain magnetic field
- **0.31–0.58 gauss**: the Earth's magnetic field ~ 5×10^{-5} T
- **50 gauss**: a typical refrigerator magnet
- **100 gauss**: a small iron magnet
- **2000 gauss**: a small neodymium-iron-boron (NIB) magnet
- **15,000-30,000 gauss**: a medical magnetic resonance imaging electromagnet
- 10^{12}–10^{13} **gauss**: the surface of a neutron star[3]

NMR Chemical Shift Chart



Chemical Shift - Makes measurements independent of magnetic field strength – measure frequency of sample vs. frequency of reference compound.

NMR Chemical Shift Chart



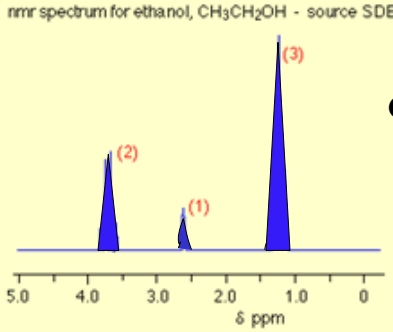
High vs. Low Resolution NMR Spectra

What a low resolution NMR spectrum tells you

Number of peaks -- number of different environments

Ratio of the areas under the peaks -- ratio of the numbers of hydrogen atoms

Chemical shifts -- environment the hydrogens



CH_3-CH_2-OH

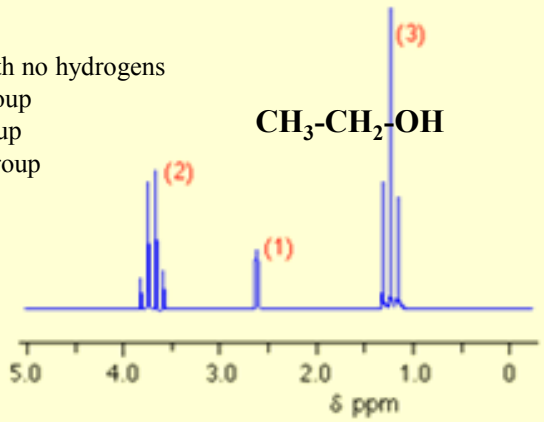
High vs. Low Resolution NMR Spectra

What a high resolution NMR spectrum tells you

In a high resolution spectrum, single peaks in the low resolution spectrum are split into clusters of peaks due to spin-spin coupling. Amount of splitting (**n+1 rule)** tells you about the number of hydrogens attached to the carbon atom ***next door.***

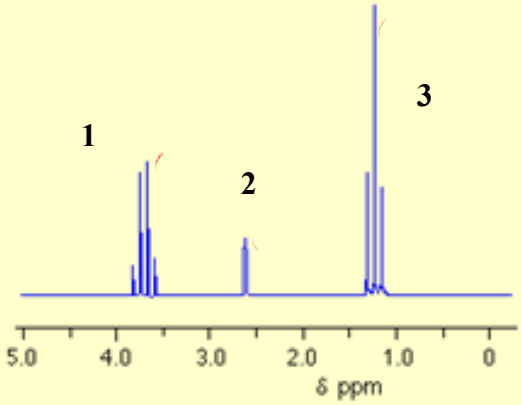
Singlet - next door to carbon with no hydrogens
Doublet - next door to a CH group
Triplet - next door to a CH_2 group
Quartet - next door to a CH_3 group

CH_3-CH_2-OH



iClicker Question 1 – November 27, 2012

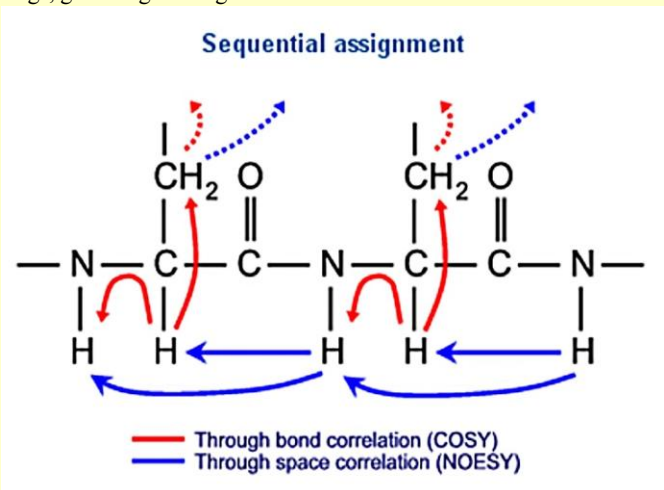
Which set of split peaks corresponds to a $-CH_2-$ group near to a methyl group?



A) 1 B) 2 C) 3 D) 1 and 3

NMR Methods – COSY vs. NOESY

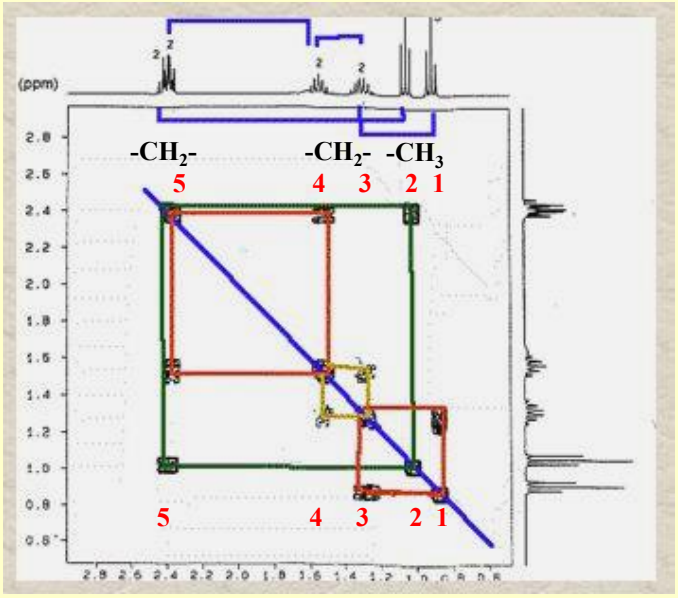
Two-dimensional **COSY** (**CO**rrelation **S**pectroscop**Y**) experiments allow you to **determine the connectivity** of a molecule by determining which protons are spin-spin coupled. One could accomplish the same task by a detailed analysis of spin-spin splittings, given high enough resolution.



http://www.bch.bris.ac.uk/staff/pfdg/teaching/nmr.htm

NMR Methods – COSY (thru bonds)

Small molecule "2D" NMR - empirical formula $C_7H_{14}O$



NOESY is a acronym for Nuclear Overhauser Effect Spectroscopy. NOE is the perturbation of the magnetization of one spin due to dipolar coupling with another spin. Since this interaction is detected through space the NOESY experiment provides important information on inter-nuclear distances.

NOE = the change in the intensity of the NMR signal of one nucleus when the sample is irradiated with radiowaves at the NMR absorption frequency of another nearby nucleus.

The NOE depends on the distance between nuclei.

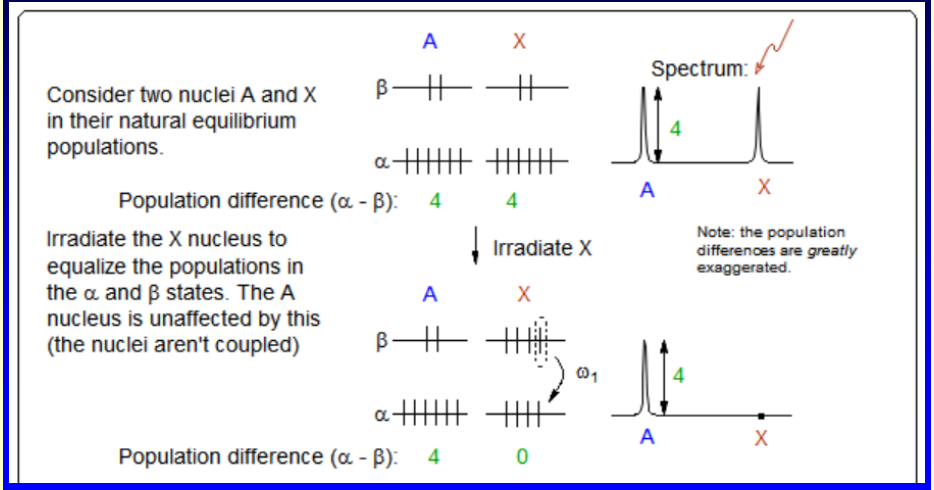
In general,

^1H to ^1H distance = 3 Å there is a large NOE

^1H to ^1H distance = 4 Å there is a medium NOE

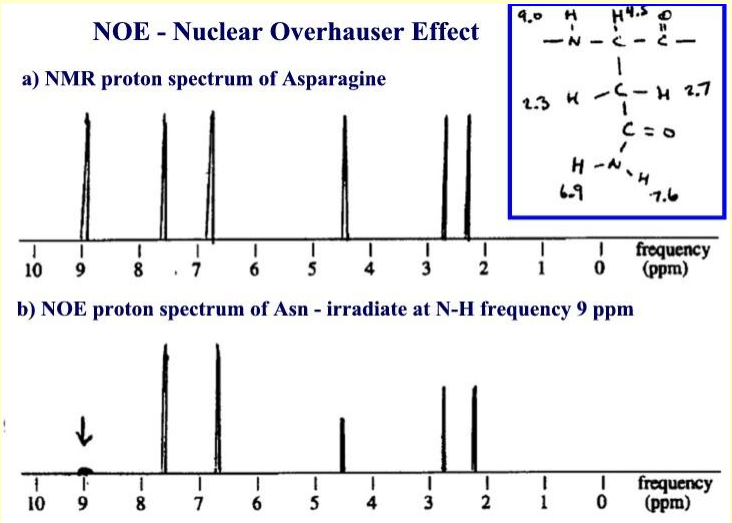
^1H to ^1H distance = 6 Å there is a small NOE

Distances from NOESY spectra:



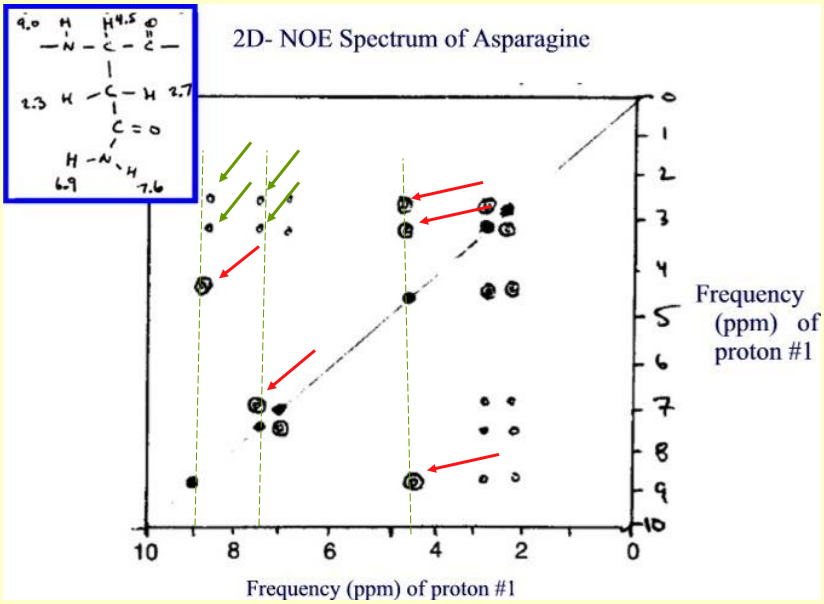
http://www.chem.wisc.edu/areas/reich/nmr/08-tech-02-noe.htm

Nuclear Overhauser Effect (NOE) - 1 Dimension



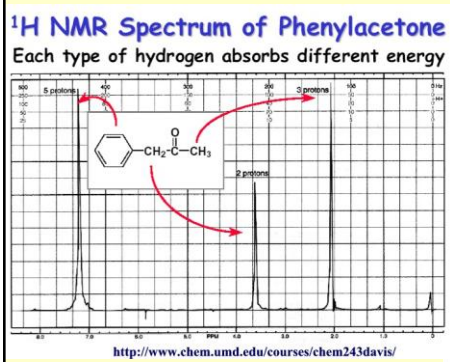
Amide –N-H (9.0) vanishes since populations have been equalized. Proton at 4.5 ppm has biggest change since it is closest.

Nuclear Overhauser Effect (NOE) – 2 Dimensions

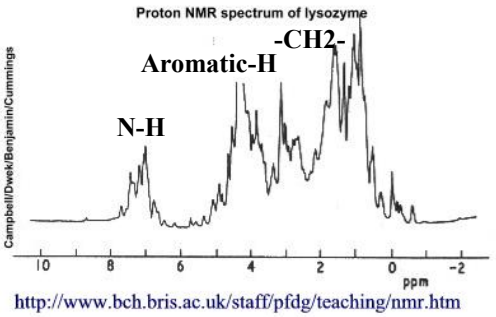


NMR Methods

Small molecule NMR



"Big" molecule NMR



Sample requirements

- ~ 0.25 ml 0.5 mM protein
  (= 2.5 mg for 20 kDa protein)
- ^{15}N, ^{13}C, (^{2}H) labelled (*E. coli*)
- MWT < ~ 60 kDa for 3D structure
- MWT < ~100 (800) kDa for secondary structure, functional tests, etc.

http://www.ti.inf.ethz.ch/ew/Lehre/GCMB07/material/lecture13/NMR.pdf

Limitations for Structure Determination by Multidimensional NMR Methods

1. **Protein must be "smallish" (< 300 amino acid residues)**
2. **Protein must be soluble and well behaved in solution.**
   (1-2 mM or 30 mg/mL for a 20kDa protein)
3. **Must be able to solve the "Assignment" Problem**

   Gly43 CA-H vs. Gly87 N-H, etc.

4 **Must have sufficient number of distance restraints**

   Gly43 CA-H / Gly87 N-H 3.0 – 4.5 A, etc.

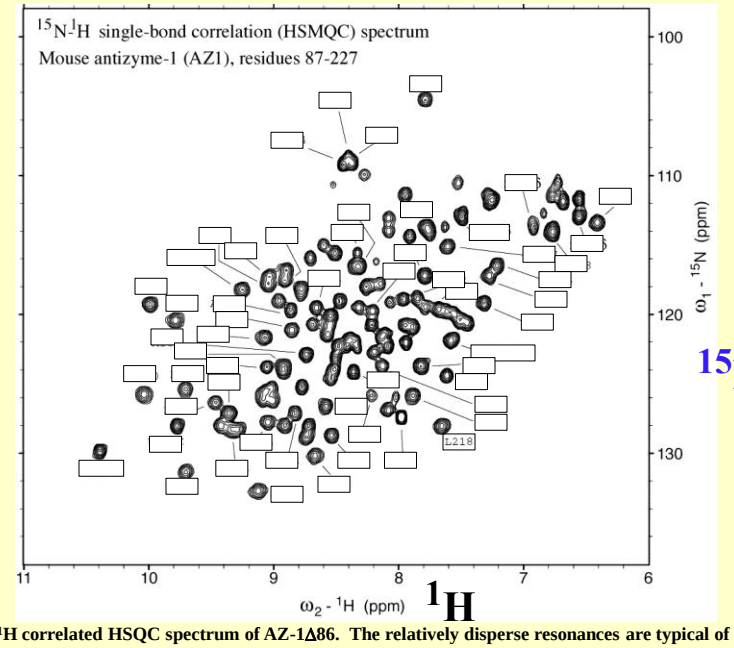
Protein Structure Determination

The determination of protein structure by NMR methods is largely based on the ability to detect and quantify **inter-proton distances** by **measurement of the dipolar coupling between protons.**

However - In order to obtain these distances it is necessary to **assign resonance frequencies to the protons with the protein**. The latter is referred to as "assigning the spectrum", or the ***assignment problem***.

The **assignments** are based largely on the detection of scalar **coupling through chemical bonds**. Analysis of spectra will give assignments of proton type (e.g. amide, alpha, beta etc.) and carbon type.

First step of NMR analysis is spectrum assignment:
(Identifying the NMR frequency of as many nuclei as possible).



The ^{15}N-^{1}H correlated HSQC spectrum of AZ-1Δ86. The relatively disperse resonances are typical of a protein that is folded and suitable for structural study by NMR methods. Some of the assigned resonances are labeled.

Resonance assignment by COSY

- COSY spectra show frequency correlations between nuclei that are connected by chemical bonds
- Since the different amino acids have a different chemical structure they give rise to different patterns in COSY spectra
- This information can be used to determine the frequencies of all nuclei in the molecule. This process is called resonance assignment
- Modern assignment techniques also use information from COSY experiments with ^{13}C and ^{15}N nuclei

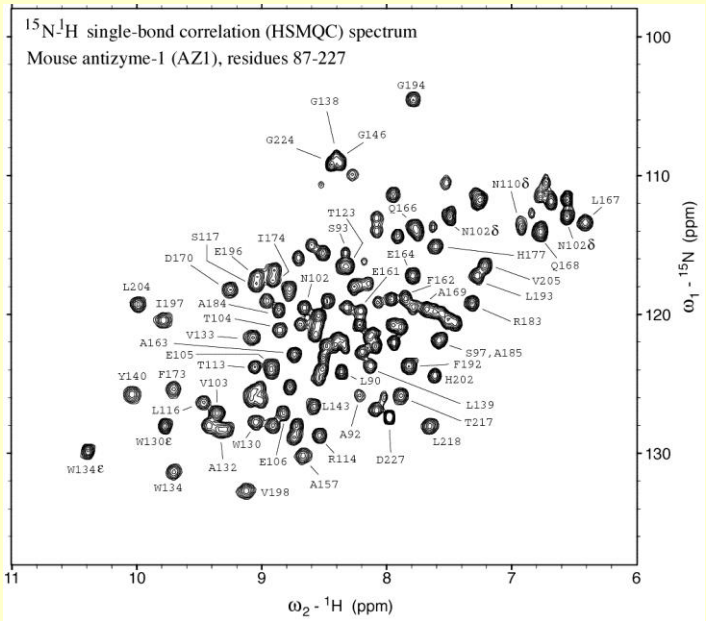
http://www.ti.inf.ethz.ch/ew/Lehre/GCMB07/material/lecture13/NMR.pdf

Proton Chemical Shifts

Residue	NH	αH	βH	Others
Gly	8.40	3.97		
Ala	8.25	4.35	1.39	
Val	8.44	4.18	2.13	0.97,0.94(CH3)
Ile	8.20	4.23	1.90	21.48,1.10 (CH2), 0.95 (γ CH3), 0.89 (δ CH3)
Leu	8.42	4.38	1.65,1.65	1.65 (γ CH), 0.94,0.90(δ CH3)
Pro	-	4.44	2.28,2.02	2.03 (γ CH2), 3.68,3.65 (δ CH2)
Ser	8.38	4.50	3.88	
Thr	8.24	4.35	4.22	1.23 (γ CH3)
Asp	8.41	4.76	2.84,2.75	
Glu	8.37	4.29	2.09,1.97	2.31,2.28 (γ CH2)
Lys	8.41	4.36	1.85,1.76	1.45 (γ CH2), 1.70 (δ CH2), 3.02 (ε CH2), 7.53 (γ NH3)
Arg	8.27	4.38	1.89,1.79	1.70 (γ CH2), 3.32 (δ CH2), 7.17,6,62 (NH)
Asn	8.75	4.75	2.83,2.75	7.59,6.91 (δ NH2)
Gln	8.41	4.73	2.13,2.01	2.38 (γ CH2), 6.87,7.59 (γ NH2)
Met	8.42	4.52	2.15,2.01	2.64 (γ CH2), 2.13 (ε CH3)
Cys	8.31	4.69	3.28,2.96	
Trp	8.09	4.70	3.32,3.19	7.1-7.5 (aromatic), 10.22 (NH)
Phe	8.23	4.66	3.22,2.99	7.3-7.4 (aromatic)
Tyr	8.18	4.60	3.13,2.92	6.85-7.15 (aromatic)
His	8.41	4.63	3.26,3.20	7.14-8.12 (aromatic)

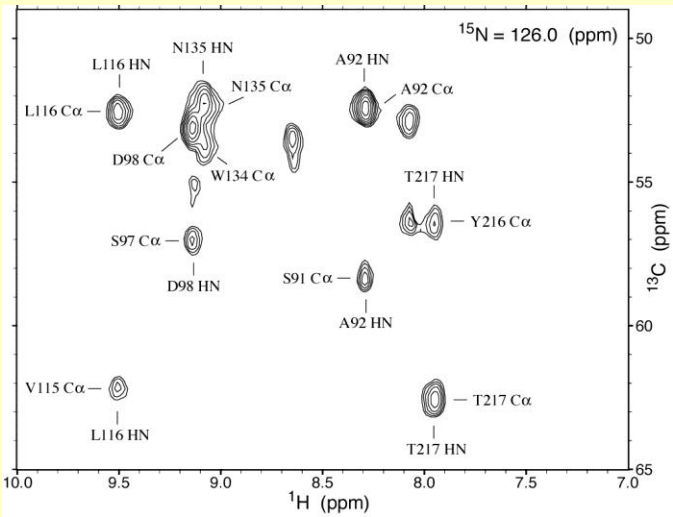
Carbon Chemical Shifts

Residue	αC	βC	Others of Distinction
Gly	45	-	
Ala	53	18	
Val	60	33	20 (CH3)
Ile	58	38	18 (γ CH3), 14 (δ CH3)
Leu	53	40	25 (δ CH3)
Pro	60	30	50 (δ CH2)
Ser	58	65	
Thr	62	70	18 (γ CH3)
Asp	55	35	
Glu	55	28	
Lys	53	32	40 (ε CH2)
Arg	55	30	42 (δ CH2)
Asn	55	35	
Gln	55	32	
Met	55	35	16 (ε CH3)
Cys	55	35(ox)/25(red)	
Trp	55	28	90-110 (aromatic)
Phe	55	35	115-125 (aromatic)
Tyr	55	35	95 (ε C), 125 (δ C)
His	55	28	100 (δ 2), 130 (ε 1)



The ^{15}N-^{1}H correlated HSQC spectrum of AZ-1Δ86. The relatively disperse resonances are typical of a protein that is folded and suitable for structural study by NMR methods. Some of the assigned resonances are labeled.

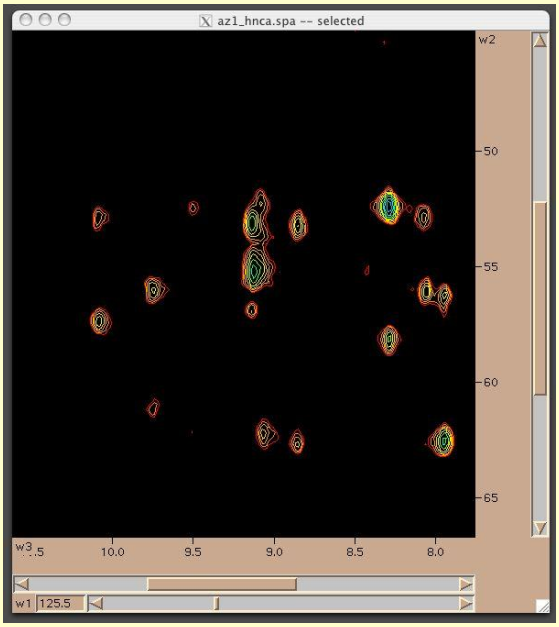
3-dimensional "triple-resonance" NMR
is used for solving the **assignment problem**.



3-D HNCA spectrum of the mouse antizyme (AZ-1Δ86). A plane corresponding to a single 15N resonance frequency is shown, obtained using our 500 MHz cryo-probe equipped instrument. Resonance peaks correlate the backbone amide ^{1}H and ^{15}N nuclei with the alpha 13C of within the same and preceeding amino acid.

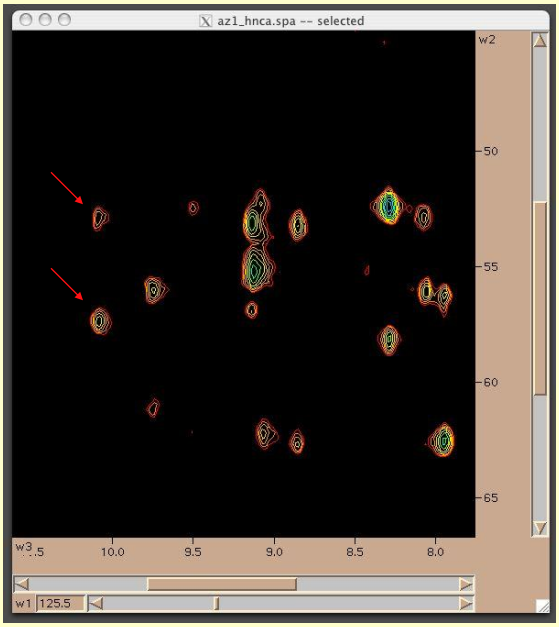
Assignments: AZ-1

"HNCA" spectrum.



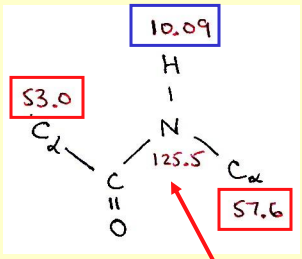
Assignments: AZ-1

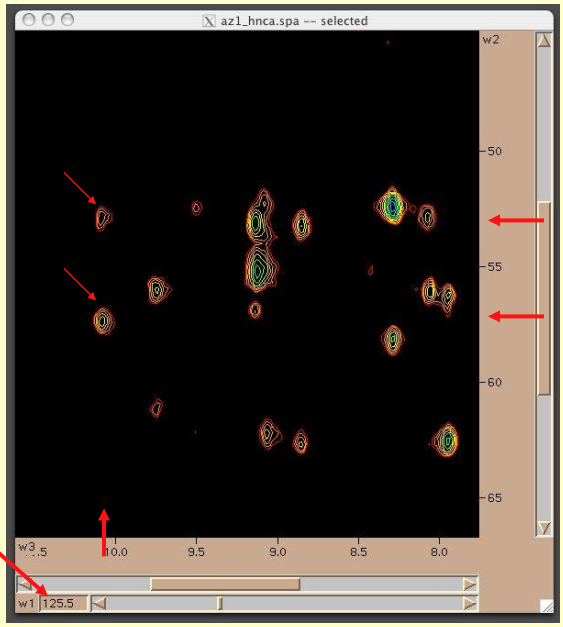
"HNCA" spectrum.



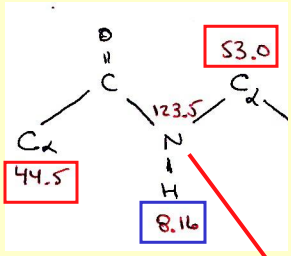
Assignments: AZ-1

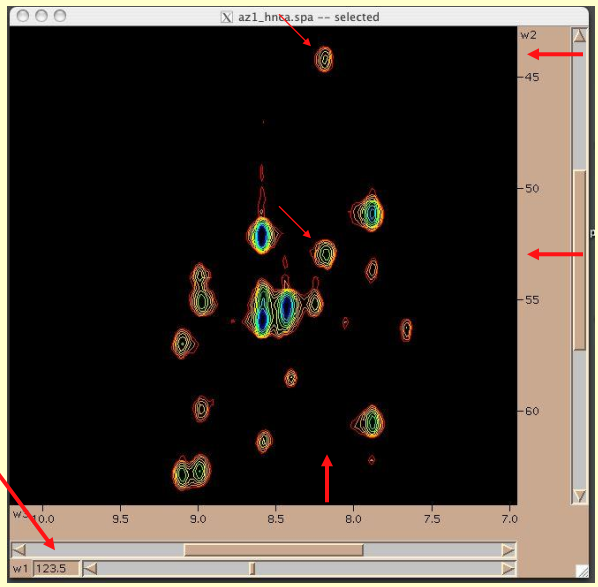
"HNCA" spectrum.



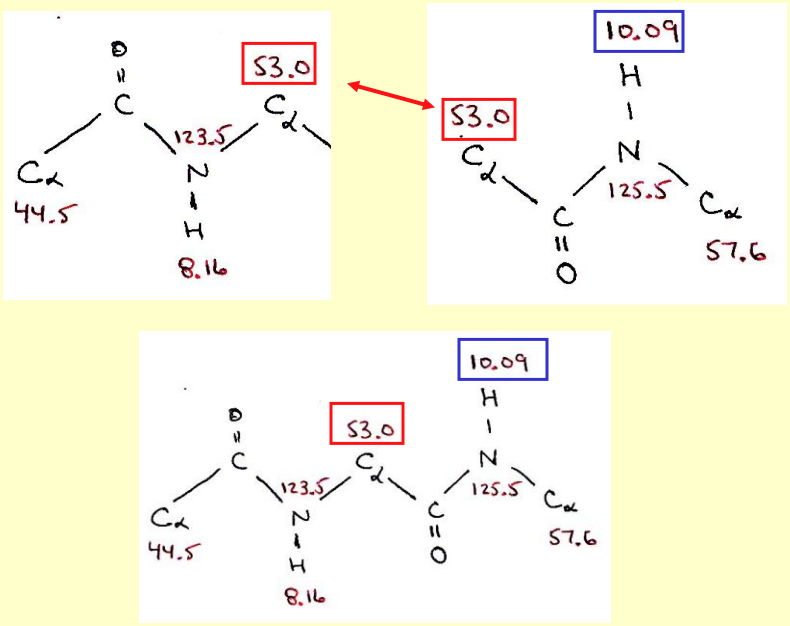


Assignments: AZ-1



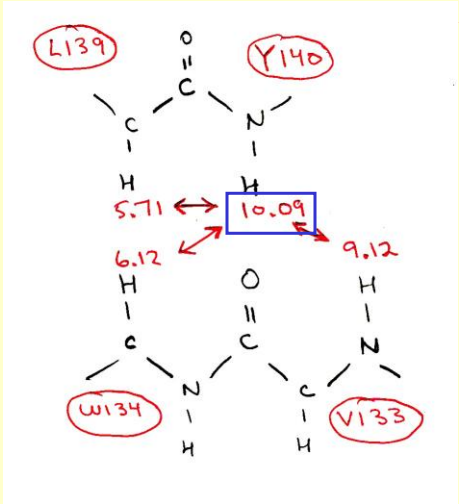


Assignments: AZ-1



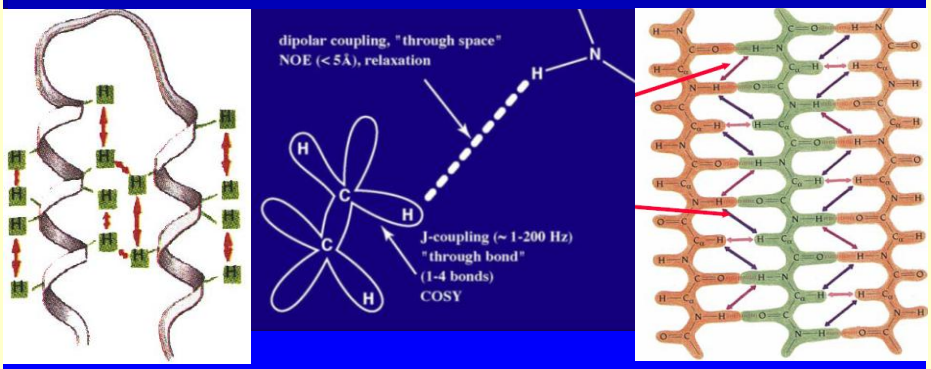
Distance Restraints:

AZ-1



Distances from NOESY spectra:

- secondary structure elements
- calculation of three-dimensional structure



http://www.ti.inf.ethz.ch/ew/Lehre/GCMB07/material/lecture13/NMR.pdf

Types of restraints available from NMR experiments

1. NOEs give rough distances between assigned atoms - given as upper and lower bounds.

2. COSY spectra and J-couplings give dihedral angle restraints

Also have constraints from what you know about the protein:

1. Connectivity due to known aa geometry & sequence

2. Standard bond lengths and angles

Energy Refinement

$$E_{TOTAL} = E_{EMPIRICAL} + E_{EFFECTIVE}$$

$$E_{EFFECTIVE} = E_{XREF} + E_{NOE} + E_{HARM} + E_{CDIH} + E_{NCS} + E_{DG} + E_{RELA} + E_{PLAN}$$

$$E_{EMPIRICAL} = \Sigma^{N}_{p=1} [w^{p}_{BOND}E_{BOND} + w^{p}_{ANGL}E_{ANGL} + w^{p}_{DIHE}E_{DIHE} + w^{p}_{IMPR}E_{IMPR} + w^{p}_{VDW}E_{VDW} + w^{p}_{ELEC}E_{ELEC} + w^{p}_{PVDW}E_{PVDW} + w^{p}_{PELE}E_{PELE} + w^{p}_{HBON}E_{HBON}].$$

Bonded Energy Terms

$$E_{BOND} = \sum_{bonds} k_b(r - r_o)^2$$

$$E_{ANGL} = \sum_{angles} (k_\theta (\theta - \theta_0)^2 + k_{ub}(r_1 3 - r_{ub})^2)$$

$$E_{DIHE} = \sum_{dihedrals\ i=1,m} k_{\varphi i} (1 + \cos(n\varphi_i + \delta_i)) \text{ if } n_i > 0$$

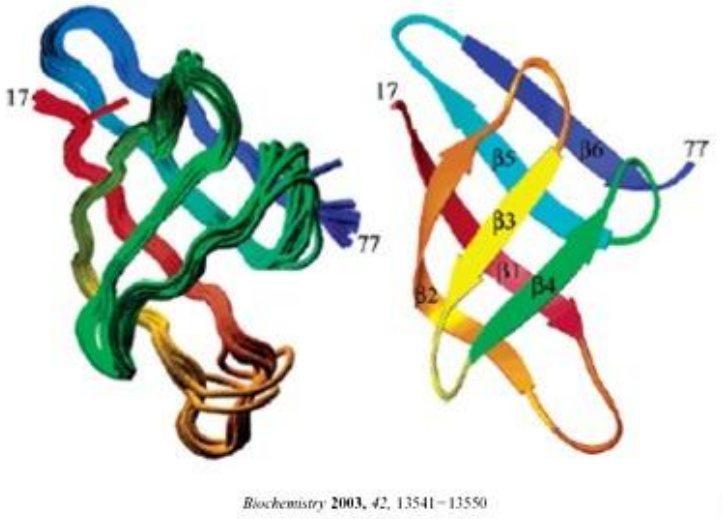
$$\sum_{dihedrals\ i=1,m} k_{\varphi i} (\varphi_i - \delta_i)^2 \text{ if } n_i = 0$$

$$E_{IMPR} = \sum_{impropers\ i=1,m} k_{\varphi i} (1 + \cos(n\varphi_i + \delta_i)) \text{ if } n_i > 0$$

$$\sum_{impropers\ i=1,m} k_{\varphi i} (\varphi_i - \delta_i)^2 \text{ if } n_i = 0$$

Table 1: Summary of Refinement and Structural Statistics for the *A. fulgidus* aRpp29 Protein (Residues 17−77)[a]

intraresidue NOEs	215
sequential NOEs (residue i to $i+1$)	178
medium-range NOEs (residue i to $i+2, 3, 4$)	18
long-range NOEs	143
dihederal angle restraints	70
hydrogen bond restraints	27
total structural restraints	651
no. of unique starting structures for simulated annealing	10
no. of simulated annealing runs, differing in initial trajectories	200
rmsd for backbone atoms (residues 17−77)	0.87 Å
rmsd for side chain atoms (residues 17−77)	1.78 Å
av no. of NOE violations >0.2 Å (per structure)	3.2 ± 1.0
av no. of NOE violations >0.5 Å (per structure)	0
residues in most favored regions of the Ramachandran plot	71.2 ± 2.6%
residues in additionally allowed regions of the Ramachandran plot	21.2 ± 4.6%
residues in generously allowed regions of the Ramachandran plot	5.8 ± 2.7%
residues in disallowed regions of the Ramachandran plot	1.9 ± 0.9%
rmsd for covalent bonds	0.0034 ± 0.0001
rmsd for covalent angles	0.511 ± 0.015
rmsd for improper angles	0.581 ± 0.016



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NMR Structure of an Archaeal Homologue of Ribonuclease P Protein Rpp29

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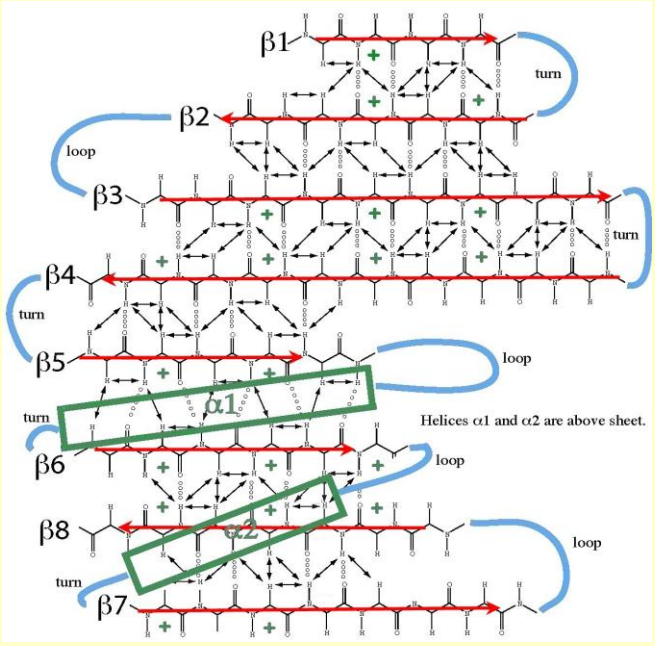
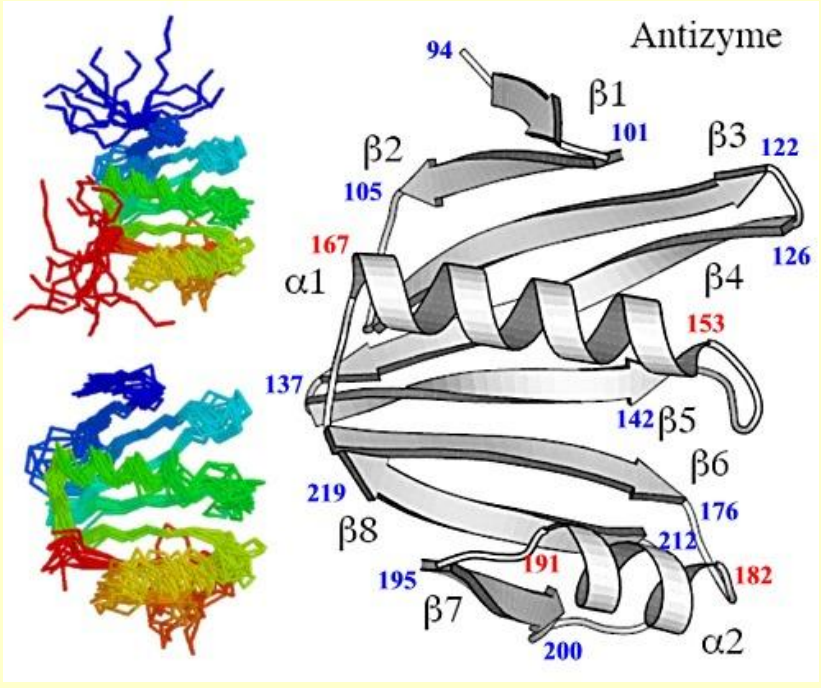
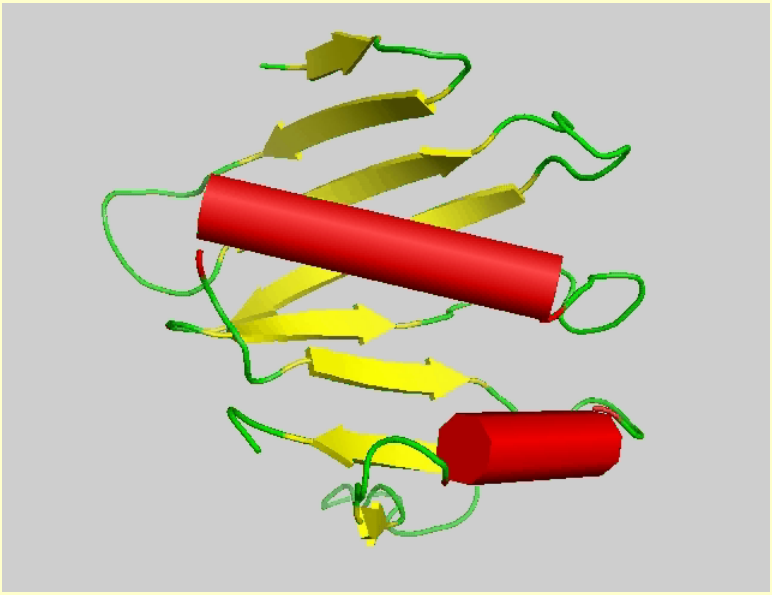
Secondary Structure of Antizyme Fragment







94I LYSDERLNVTEEPTSNDKTRVLSLQCTLTEAKQVTFRAVWNGGGLYGEDVPAGPLPESKDSF
AALLEGAEEQLRADHVFTCLPKNREDRAALLRTLSSFLGFEIVRPGHPLVPKRPDACFMVYTLE219

Summary: How are NMR structures solved?

1. **Solution phase technique** - protein at mM concentration in a buffer. Currently limited to proteins ≤ 30-50 kDa.
2. **Measure resonant frequencies** of ^{1}H, ^{13}C, ^{15}N atoms in a magnetic field.
   1D, 2D, 3D NMR
3. **Assign peaks** observed in the spectrum to individual amino acids.
   COSY
4. **Measure distances** between different residues < 6Å apart to get restraints. Need many restraints per residue.
   NOESY
5. **Build structures** consistent with the experimental distance restraints and principles of sterochemistry.
   Simulated Annealing
6. Yields a **set of structures** consistent with the data.
   Blur-o-gram

Comparison of X-ray vs. NMR Structure Determination

a) Limitations.

X-ray: Need crystals
NMR: MW limit (over about 40 kDa spectra are too complex to interpret)

b) Ease of structure determination.

c) Quality of structural information obtained.

X-ray: Usually has the advantage, especially with high-resolution structures, due to direct visualization of the molecule.

NMR: Very good quality structures are also obtained, though usually not as detailed as the best x-ray structures. Quality of the NMR structure depends on the # of distance and angle constraints obtainable from the data.

d) NMR has some advantages over x-ray crystallography:

Information may be obtained on the dynamics of structures. Such as hydrogen bond opening frequencies, & rotational times of bond vectors.