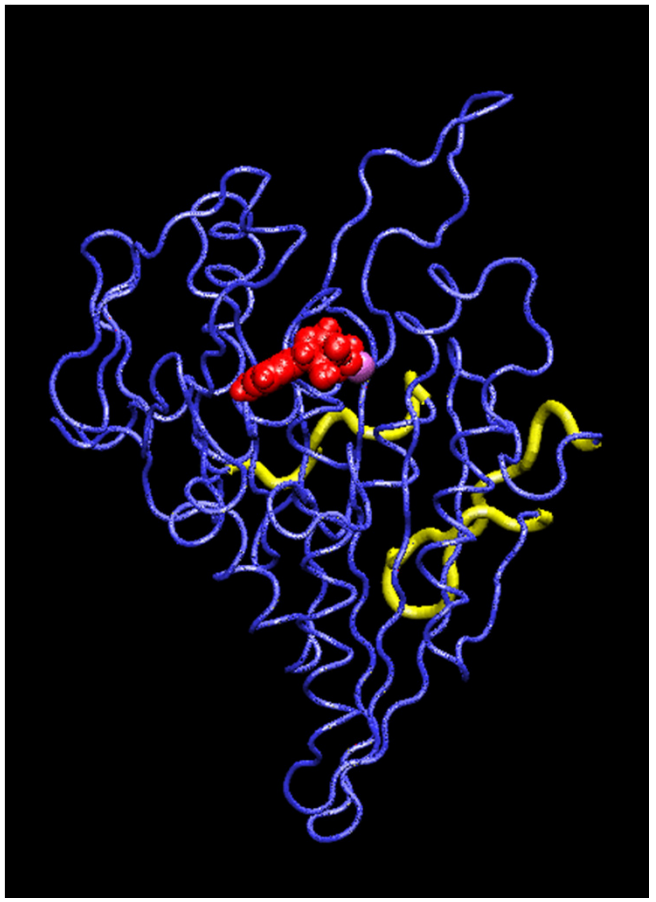
NMR Analysis of Protein-Ligand Interactions

A Ligand Interaction with a Protein will Perturb Both Structures

- These structural perturbations are reflected by changes in a variety of NMR physical parameters or observables including:
  - chemical shifts
  - relaxation parameters T_1,T_2(line-width) and NOEs
  - dynamic parameters ($S^{2,}$ H/D exchange)
  - diffusion coefficients
  - saturation transfer difference
  - transfer NOE
- Solve a Protein-Ligand co-structure

Conformational changes induced in the kinesin structure (blue) by the additional gamma phosphate (green) of ATP

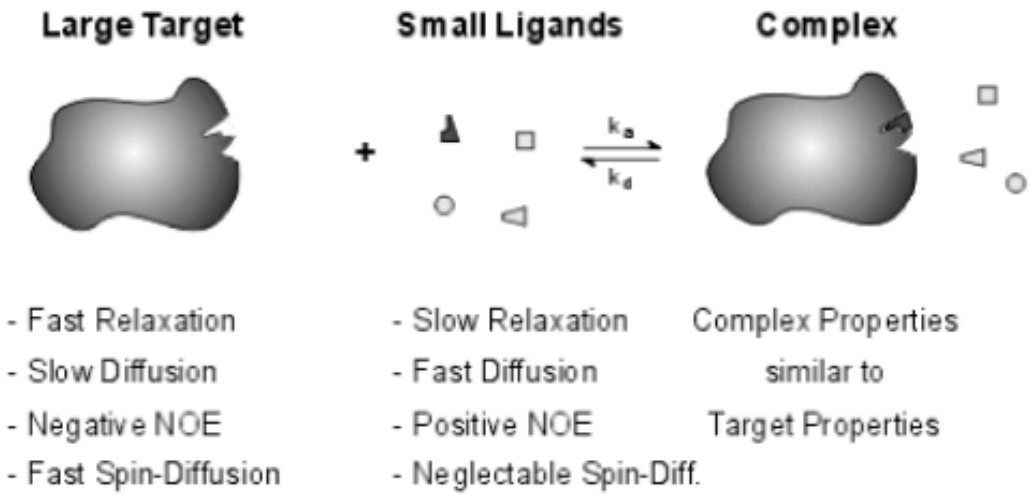


Can Monitor Either Ligand or Protein Changes

DSMM - Database of Simulated Molecular Motions
http://projects.villa-bosch.de/dbase/dsmm/

NMR Analysis of Protein-Ligand Interactions

NMR Monitors the Different Physical Properties That Exist Between a Protein and a Ligand

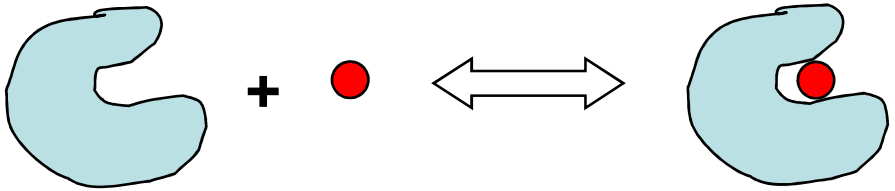


NMR Analysis of Protein-Ligand Interactions

Ligand Line-Width (T_2) Changes Upon Protein Binding

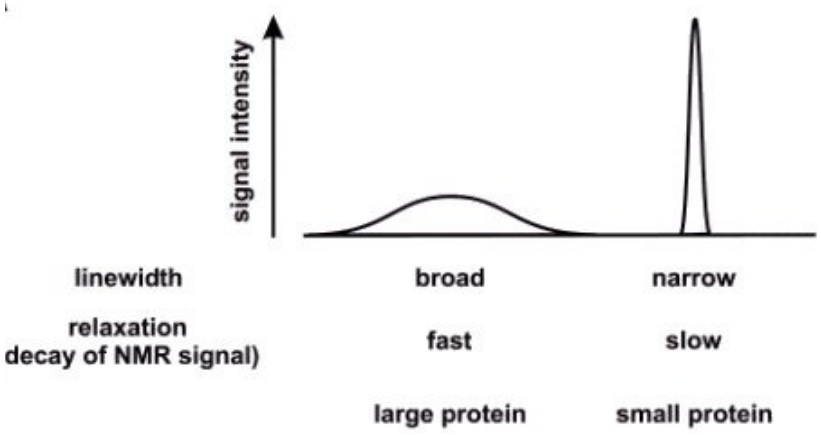
- As we have seen before, line-width is directly related to apparent MW
  - a small-molecule (~100-1,000Da) is orders of magnitude lighter than a typical protein (10s of KDa)
    - a small molecule has sharp NMR line-widths (few Hz at most))
    - protein has broad line-widths (10s of Hz)
  - if a small molecule binds a protein, its line-width will resemble the larger MW protein

$\tau_c \approx MW/2400$ (ns)



Small molecule:

Sharp NMR lines **Broad NMR lines**

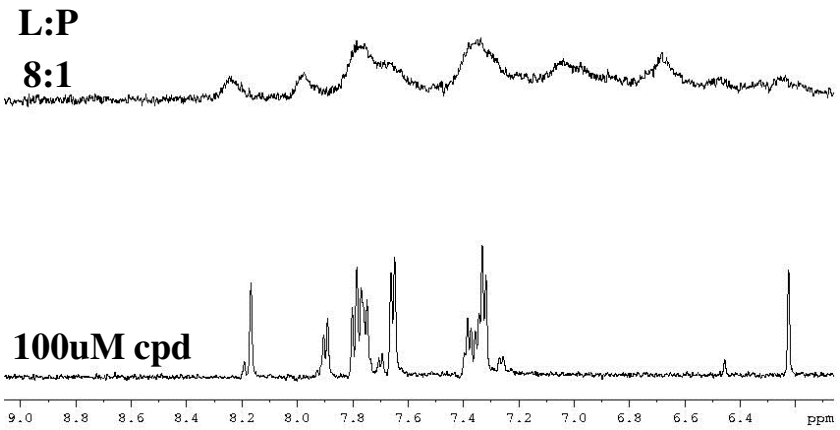


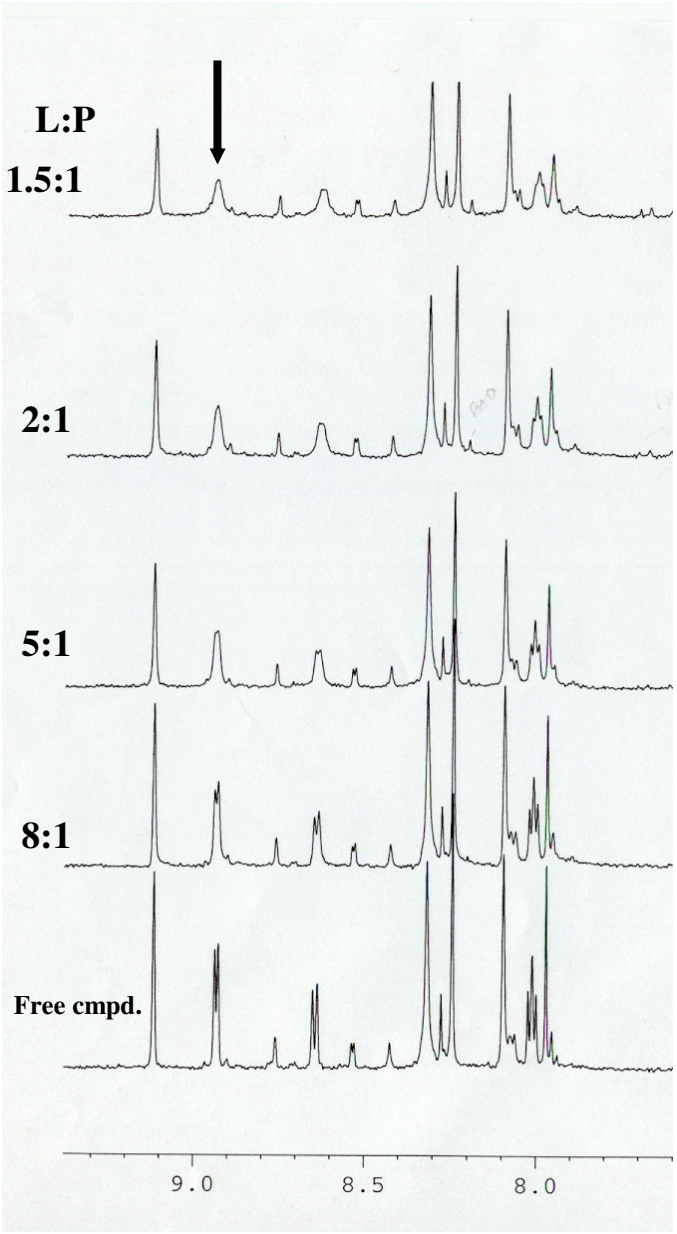
NMR Analysis of Protein-Ligand Interactions

Ligand Line-Width (T_2) Changes Upon Protein Binding

- As a protein is titrated into a ligand NMR sample, the ligands line-width will broaden if it binds the protein

Dramatic increases in line-width at low protein concentrations may indicate multiple non-specific binding

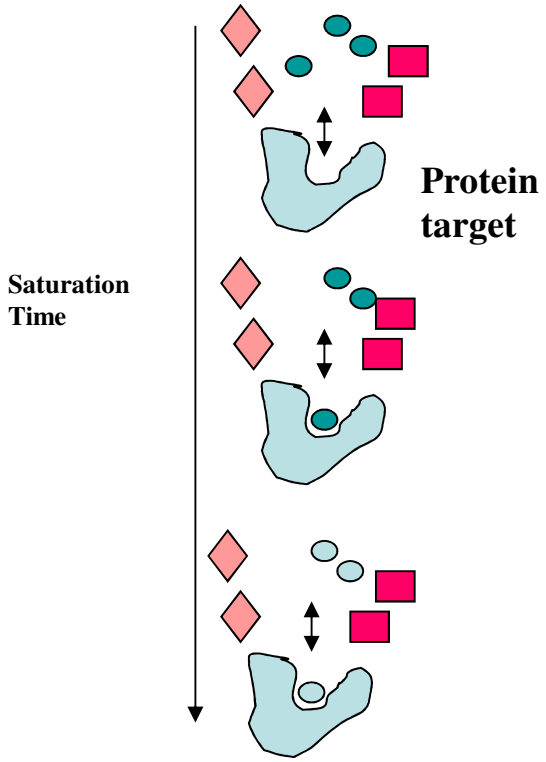


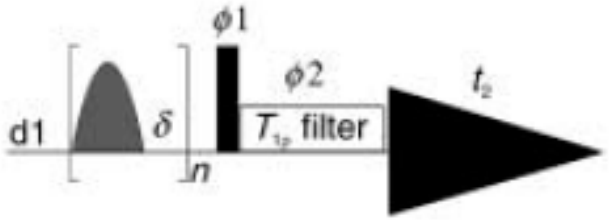


NMR Analysis of Protein-Ligand Interactions

Saturation Transfer Difference (STD)

- Selectively irradiate protein resonances
  - saturation pulse of 1-2 sec
  - chain of Gaussian pulses of 50 ms duration separated by 1ms
- Small molecules that bind will also be saturated
  - small molecule is 20-30 fold excess
- record difference spectrum
  - 1st spectra on-resonance (typically -0.4 ppm)
  - 2nd spectra off-resonance (typically 30 ppm)
  - only binders will exhibit NMR spectra
  - ligands relax by normal T_1/T_2 process





Gaussian envelope (selective irradiation)

$$S(t) = \exp[-a(t-t_0)^2]$$

where:

t_o - center of the pulse envelop
S - intensity of the pulse
a - pulse duration (pulse width)
t - time.

Angew. Chem. Int. Ed. 2003, 42, 864 – 890

NMR Analysis of Protein-Ligand Interactions

Saturation Transfer Difference (STD)

- Saturation transfer occurs during the duration of the selective saturation pulse (τ_{sat})
  - ♦ during this time period (1-2 sec) multiple ligands (*n*) bind the protein that depends on the off-rate (k_{off})

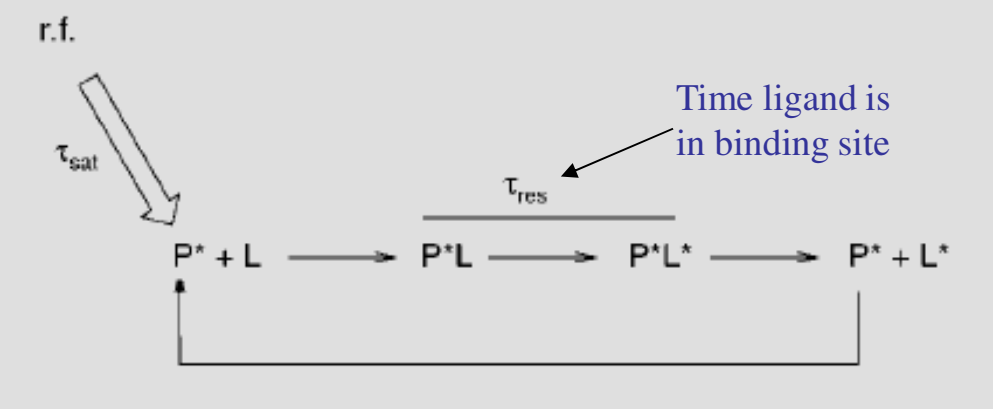
$$P + L \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} PL \qquad K_D = \frac{[P][L]}{[PL]} = \frac{k_{off}}{k_{on}}$$

Table 1:

K_D	k_{off} [s^{-1}]
1 mM	10 000
1 µM	10
1 nM	0.01

  - ♦ weaker binding → higher k_{off} → stronger STD signal
  - ♦ larger the number of ligands (*n*) that bind during τ_{sat}

$$n = f_{PB} * t_{sat} / t_{res}$$

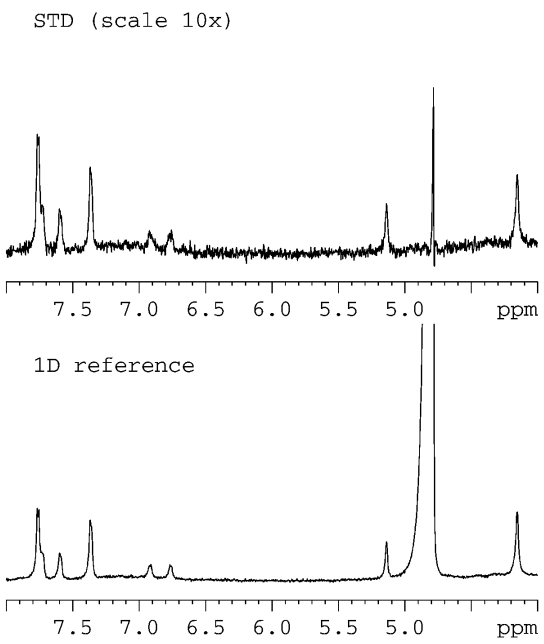


  - ♦ tight binding ligands (kD ≤ 1 nM) no STD signal, too slow an off-rate

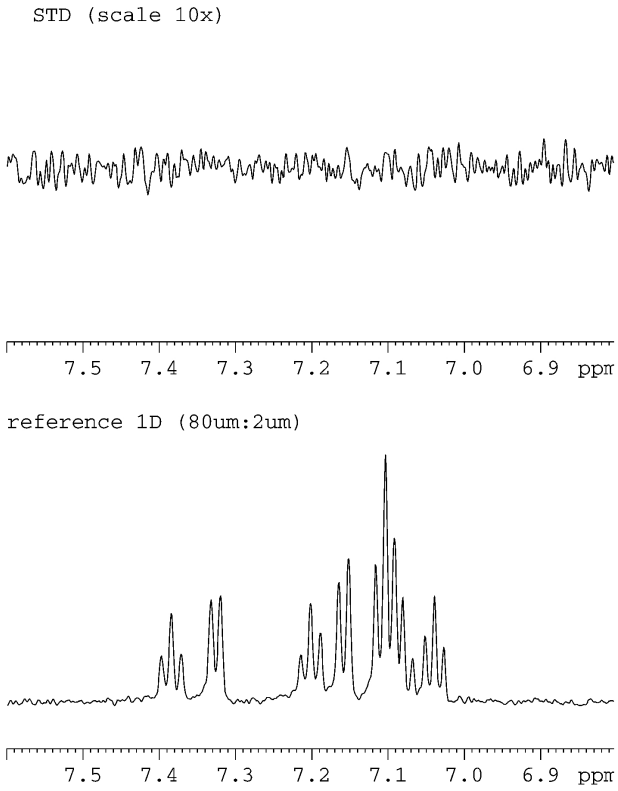
NMR Analysis of Protein-Ligand Interactions

Saturation Transfer Difference (STD)

Binder



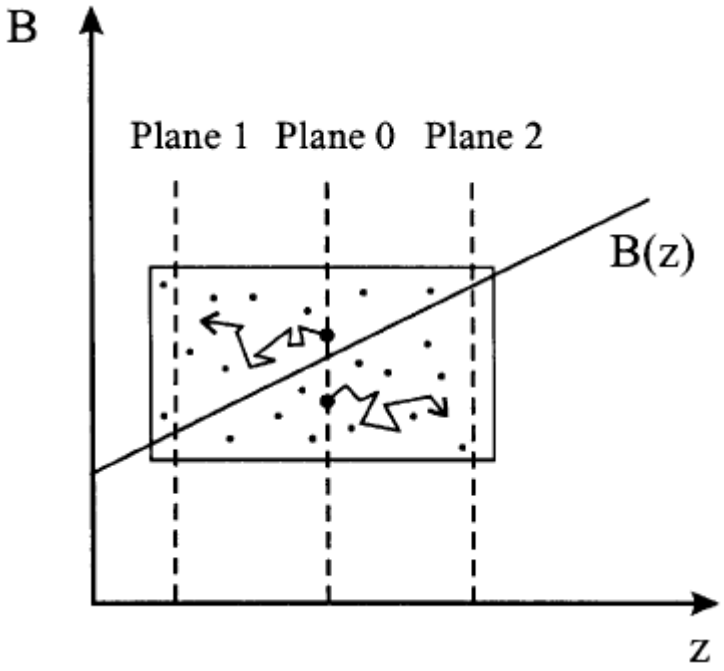
Non-Binder



WATER-LOGsy – variant of STD where saturation transfer involves bound water instead of protein i.e. saturate water resonance

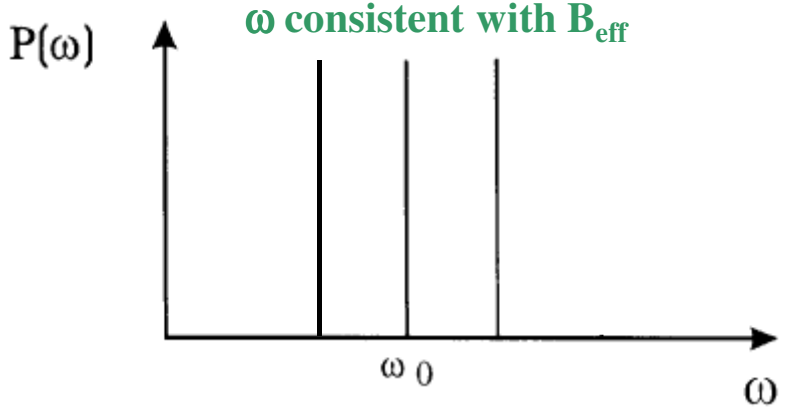
NMR Analysis of Protein-Ligand Interactions

Use of Diffusion to Identify Ligand Binding



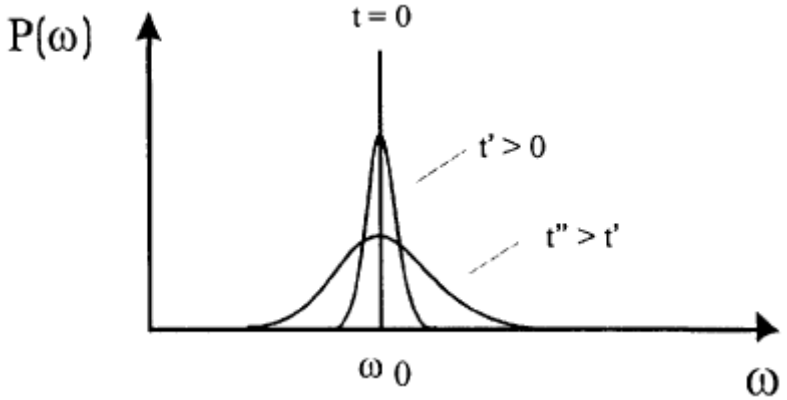
Effective field strength (B_{eff}) is different at each plane because of varing field gradient (B_z)

No diffusion



resonant at different ω consistent with B_{eff}

Diffusion

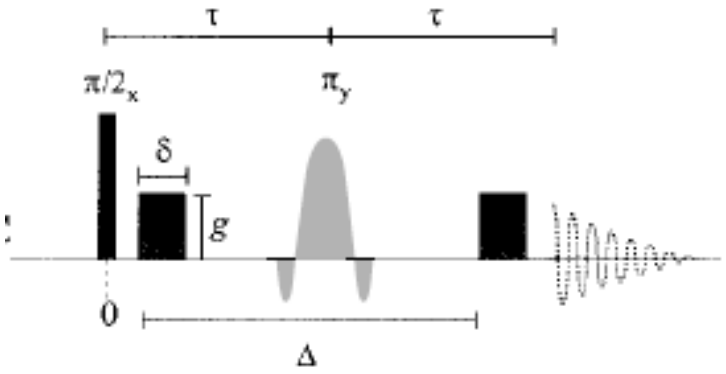


molecule randomly moves through different B_{eff}, broad range of ω

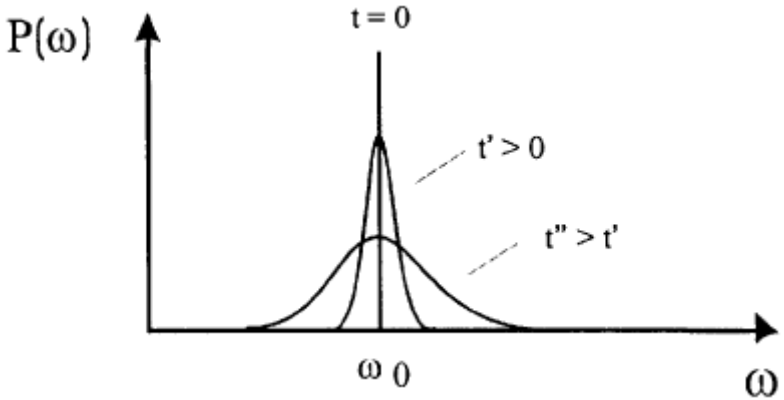
Annu. Rep. Prog. Chem., Sect. C, **2002, 98, 121–155**

NMR Analysis of Protein-Ligand Interactions

Use of Diffusion to Identify Ligand Binding



$$E = \frac{S(g)}{S(0)} = \exp\left[-\gamma^2 g^2 D \delta^2 (\Delta - \delta/3)\right]$$



Strength of signal is dependent on rate of diffusion and length/strength of gradient pulse

$$PL \underset{}{\overset{K_d}{\rightleftharpoons}} P + L$$

$$D = (1 - P_b)D_f + P_b D_b$$

Observed Ligand diffusion is the populate-weighted average of the free and bound diffusion

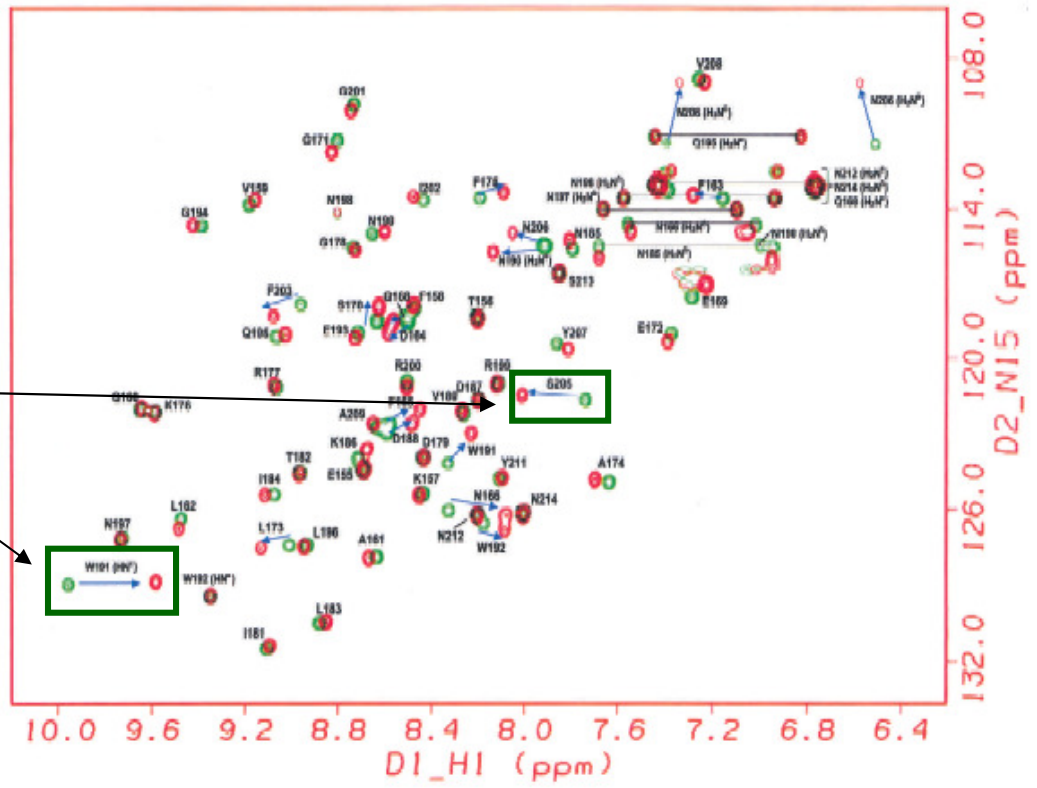
Magn. Reson. Chem. **2002; 40: 391–395**

NMR Analysis of Protein-Ligand Interactions

Protein Chemical Shift Changes Upon Ligand Binding

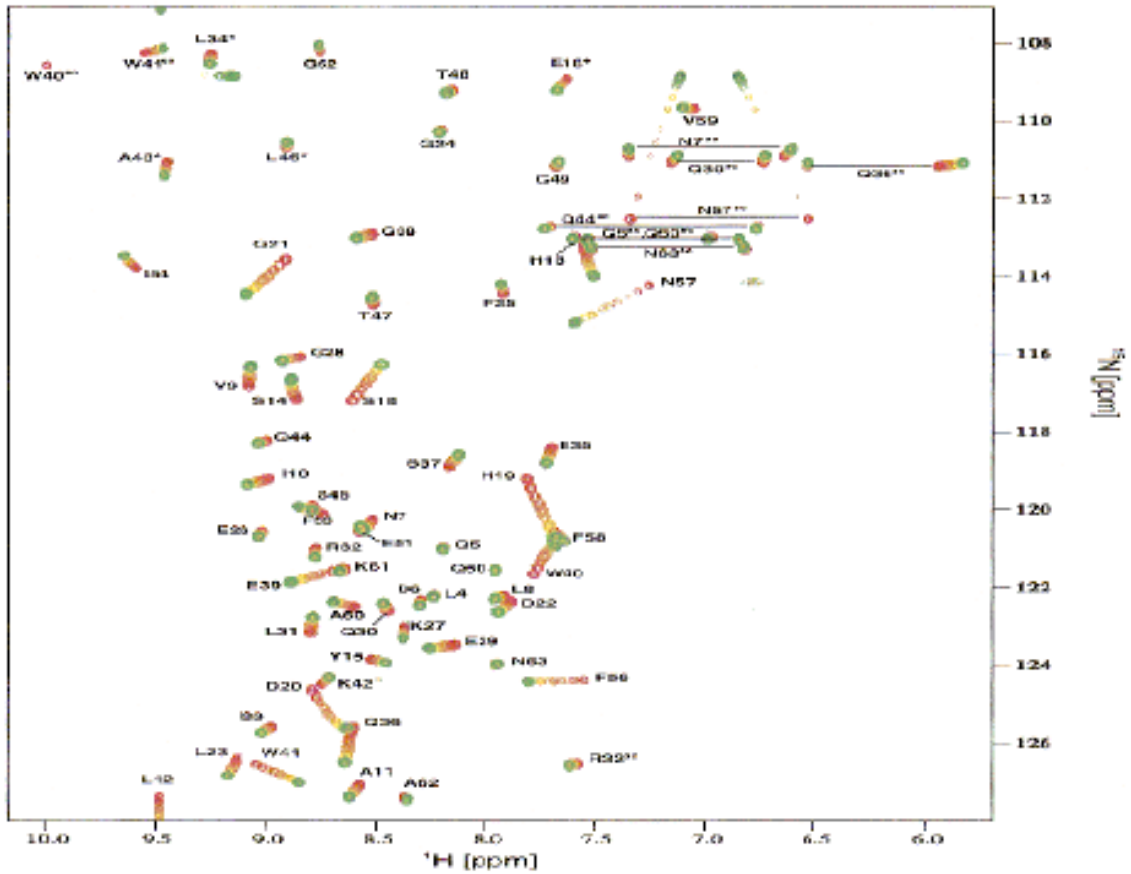
- Assigned 2D ^{1}H-^{15}N HSQC NMR Spectra
  - overlay spectra in presence/absence of ligand
  - changes in peak position indicate binding
  - identity of peaks that change identifies binding site on protein surface
    - if a defined residue cluster is not observed → non-specific binding
    - if a majority of the peaks incur changes →detrimental interaction such as unfolding or aggregation

Peptide Binding to C-terminal SH3 domain of Sem-5 induces chemical shift changes



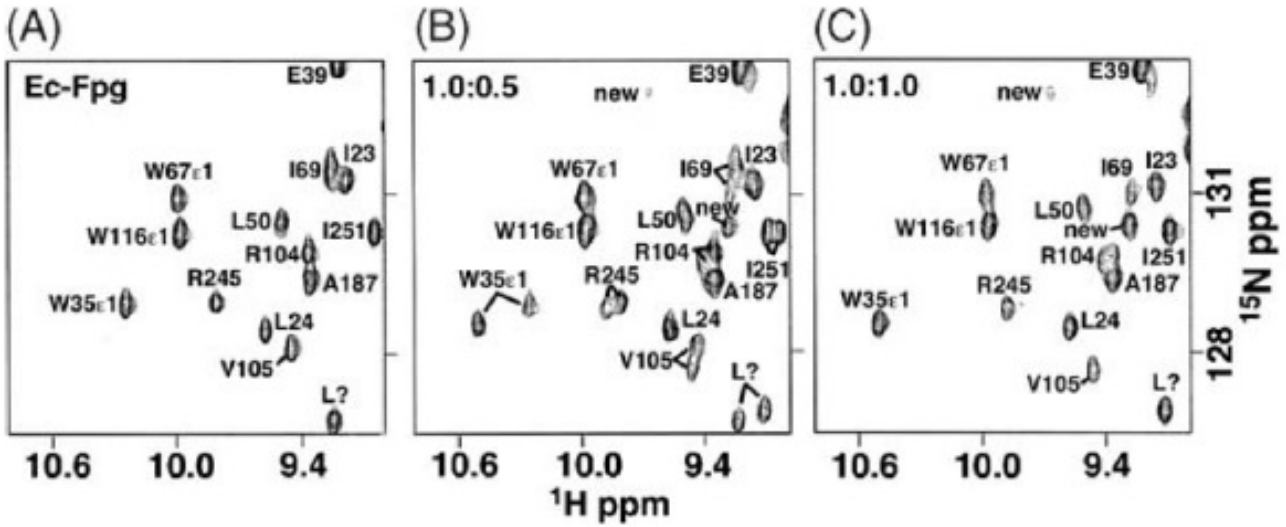
Protein Science (2003), 12:982–996.

Example of ^{1}H-^{15}N HSQC Chemical Shift Titration Under Conditions of Fast Exchange



NMR titration experiment showing the changes in the ^{1}H-^{15}N HSQC spectrum of free LckSH3 (red) upon gradual addition of Tip(173-185). Resonances belonging to the spectra after the final step of the titration (4-fold molar excess of Tip) are shown in green.

Example of ^{1}H-^{15}N HSQC Chemical Shift Titration Under Conditions of Slow Exchange

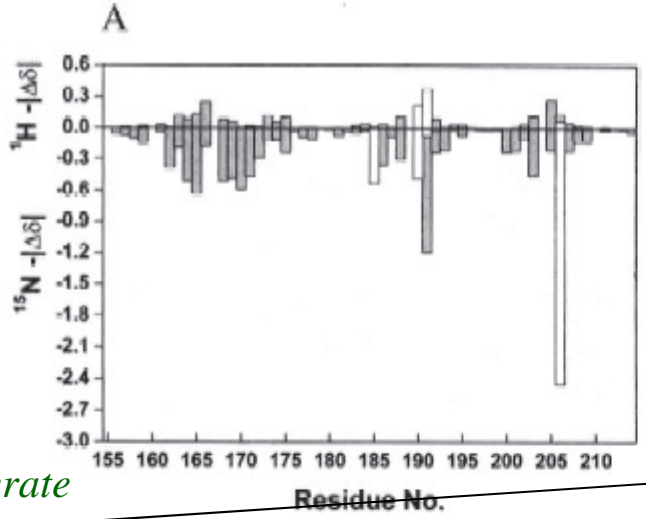


Portion of the $^{15}N/^{1}H$ HSQC spectrum of perdeuterated Ec-Fpg free in solution (A) and in the presence of approximately a 0.5 (B) and 1.0 (C) molar ratio of 13-PD. Spectra were recorded at a ^{1}H frequency of 900 MHz, 25 °C. A tight protein/DNA complex, with a K_d in the slow exchange range ($K_d > 10^{-6}$ M), is suggested by the observation of two cross peaks for a subset of the residues at the 1.0:0.5 protein-to-DNA ratio

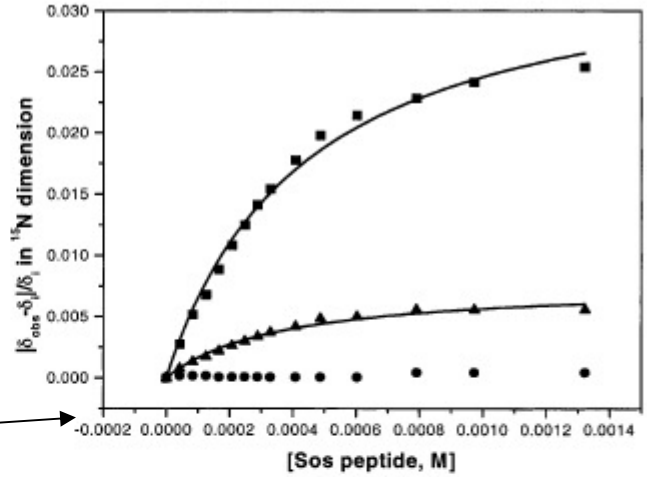
NMR Analysis of Protein-Ligand Interactions

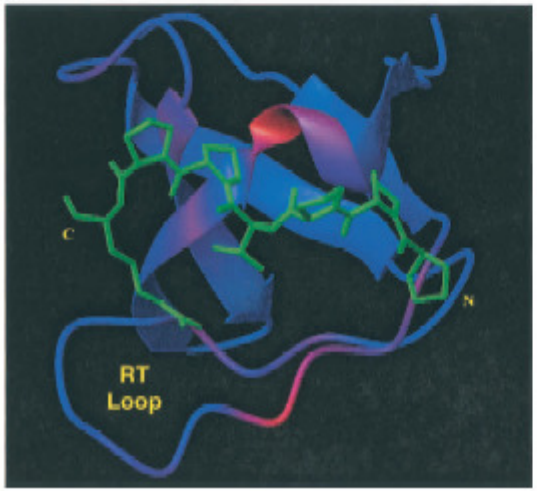
Chemical shift changes as a function of sequence identifies the major interaction sites of the ligand

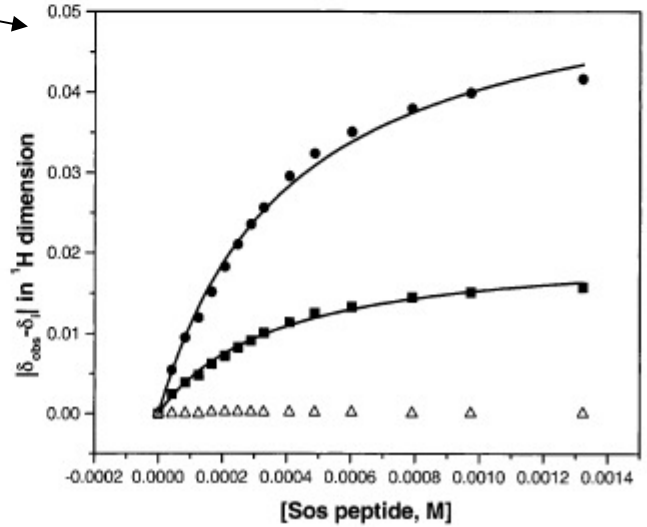
$$(\Delta\delta = \sqrt{(\delta H^2 + 0.2\ (\delta^{15}N)^2)})$$



Can be used to generate binding curves and measure K_D's





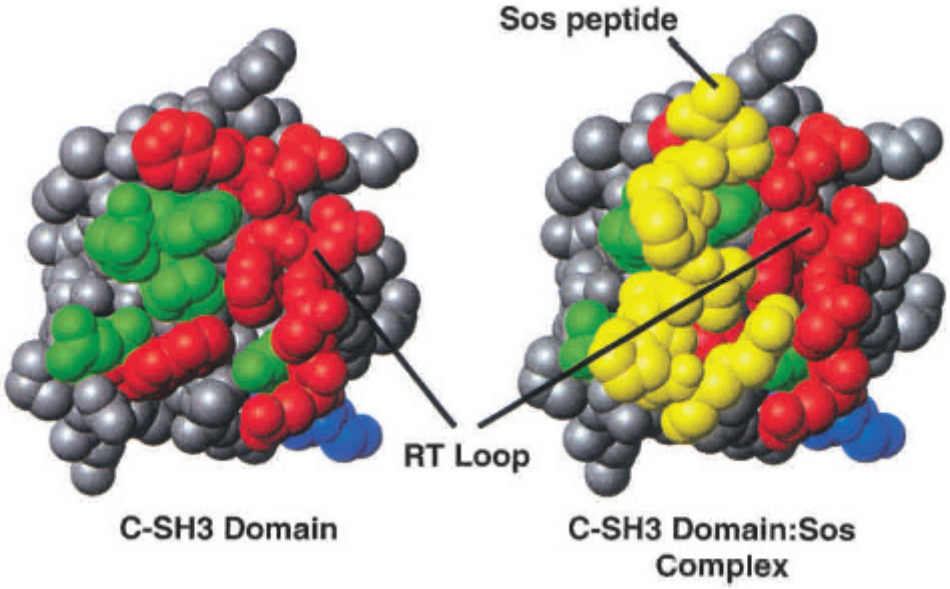


Can be compared to the structure to identify the ligand binding site

NMR Analysis of Protein-Ligand Interactions

Protein Chemical Shift Changes Upon Ligand Binding

- Visualization of Chemical Shift Changes
  - ♦ color-code residues that incur changes on protein structure



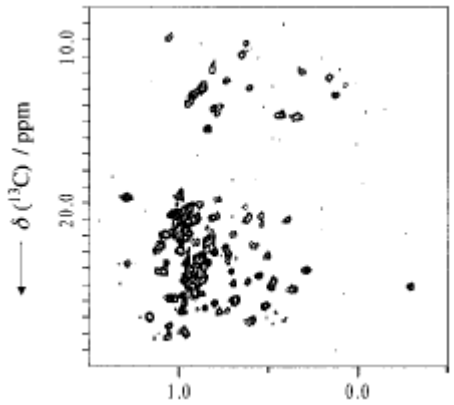
Red residues – changes in chemical shift
Green residues – no changes in chemical shifts
Blue residues – changes in chemical shift, but don't interact with peptide

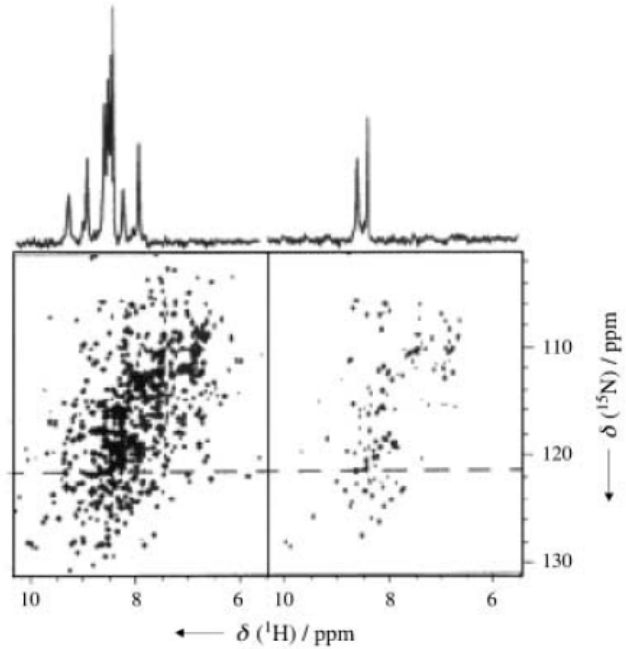
NMR Analysis of Protein-Ligand Interactions

Protein Chemical Shift Changes Upon Ligand Binding

- A Number of Perturbations to the Approach to Simplify Analysis
  - Simplify the spectra by using specific labeling
    - one residue type (Only His ^{15}N and/or ^{13}C labeled)
    - ^{13}C methyl (^{1}H-^{13}C HSQC, increase sensitivity CH_3 vs. NH)
    - spin-labeling of the protein, large chemical shift changes and line broadening occur if ligand binds near spin-label
    - ^{19}F-labeled ligands
  - TROSY with deuterium labeling for large MW proteins
  - SEA-TROSY
    - only observe surface exposed residues
    - uses a transfer from water to NHs

^{1}H-^{13}C HSQC CH_3 region of 42KDa protein



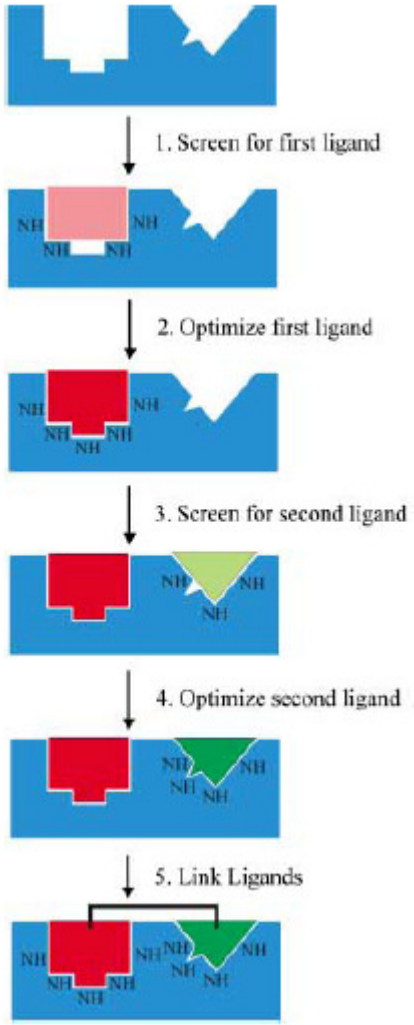


TROSY SEA-TROSY

NMR Analysis of Protein-Ligand Interactions

Number of Drug Discovery Schemes Based on Chemical Shift Perturbations

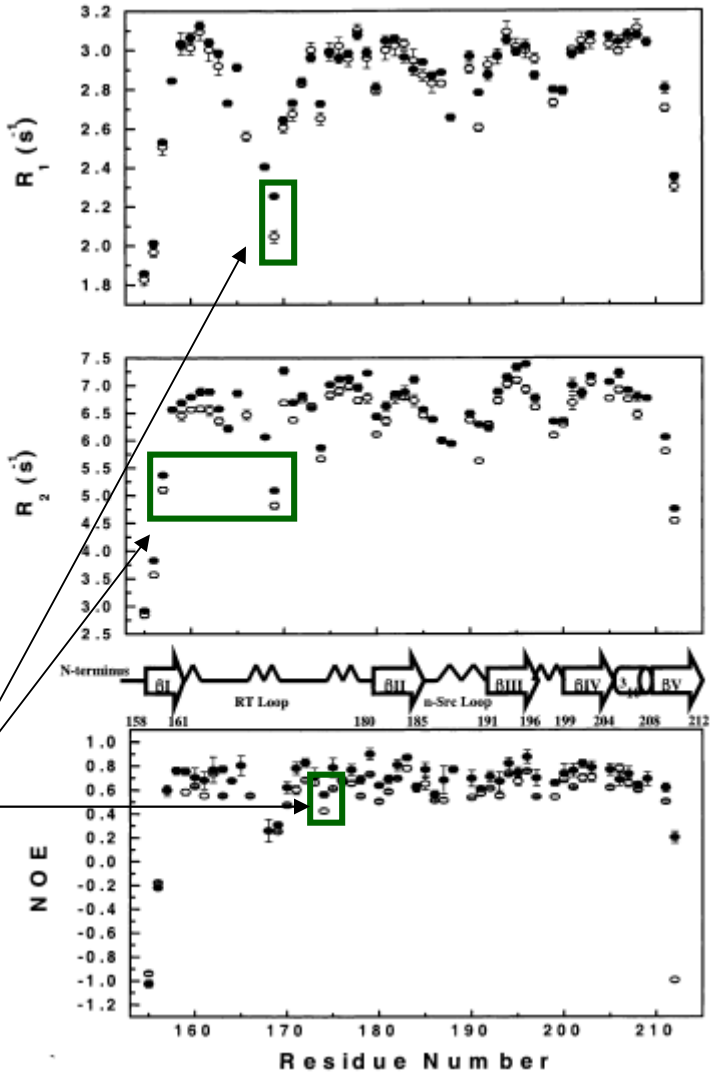
- SAR by NMR
  - Identify ligands that bind from 2D ^{1}H-^{15}N or ^{1}H-^{13}C HSQC
  - chemical shift changes
  - Identify ligands that bind close but in different binding sites
  - chemically link the two or more ligands
    - binding affinity of the linked compounds is the product of the two individual compounds
- SHAPES
  - uses a small library of drug fragments and STD NMR
- MS/NMR
  - a tiered approach combining size-exclusion chromatography (SEC), MS and NMR
  - only ligands that bind the protein pass through SEC and are detected by MS
  - collected 2D ^{1}H-^{15}N HSQC spectra only on hits from SEC-MS
- SOLVE NMR
  - target proteins with two known binding sites
  - bind a known ligand to a known binding site
  - measure NOEs from second ligand to labeled active-site residue
  - link two compounds
- RAMPED-UP NMR
  - simultaneously screen multiple proteins that are labeled differently



NMR Analysis of Protein-Ligand Interactions

Protein Mobility Changes Upon Ligand Binding

- T_1, T_2, NOE Dynamic Data
  - ♦ measure protein dynamic data in presence and absence of ligand
  - ♦ residues that exhibit significant dynamic changes indicate binding
  - ♦ identity of residues that exhibit dynamic changes identifies binding site on protein surface
    - ➢ binding of ligand usually reduces the mobility of a dynamic region of a protein



Differences in free & bound form of protein

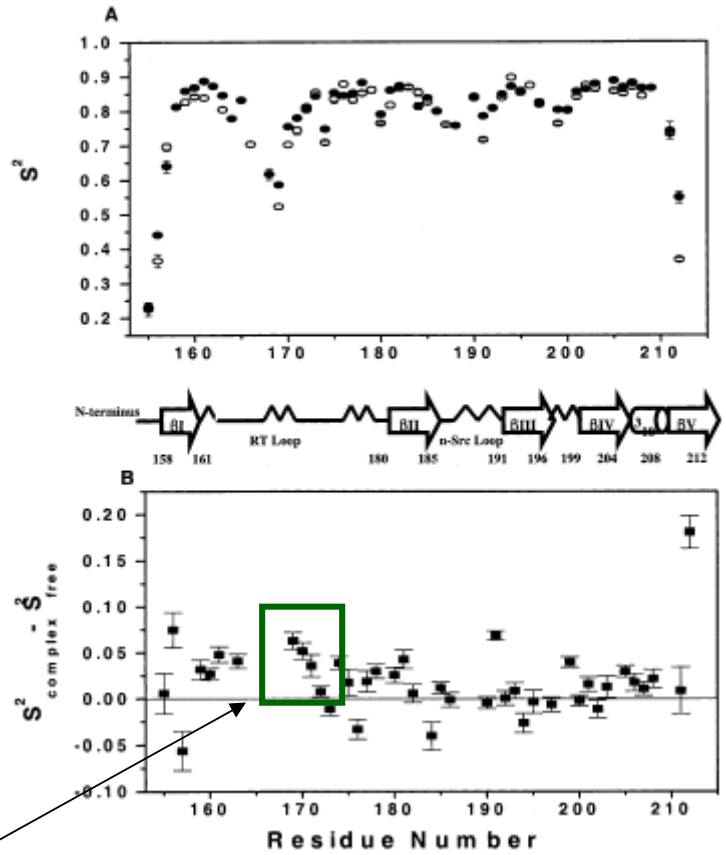
Protein Science (2003), 12:982–996.

NMR Analysis of Protein-Ligand Interactions

Protein Mobility Changes Upon Ligand Binding

- Calculated Order Parameters (S^2)
  - ♦ decrease in mobility is indicated by an increase in S^2
  - ♦ change in mobility indicates binding and defines location

Easier to identify S^2 changes by plotting difference in S^2 as a function of sequence since magnitude changes in S^2 may be small

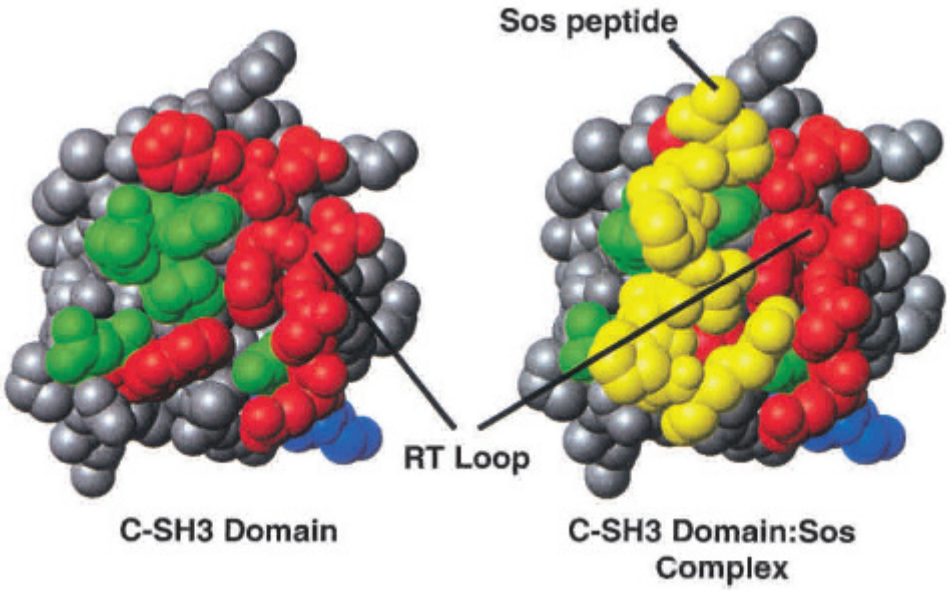


Major changes typically occur in loop regions→ site of ligand binding

NMR Analysis of Protein-Ligand Interactions

Protein Mobility Changes Upon Ligand Binding

- Map residues that incur dynamic changes onto protein surface
  - ♦ helps visualize ligand binding site
  - ♦ rationalize source of mobility change from protein-ligand interactions

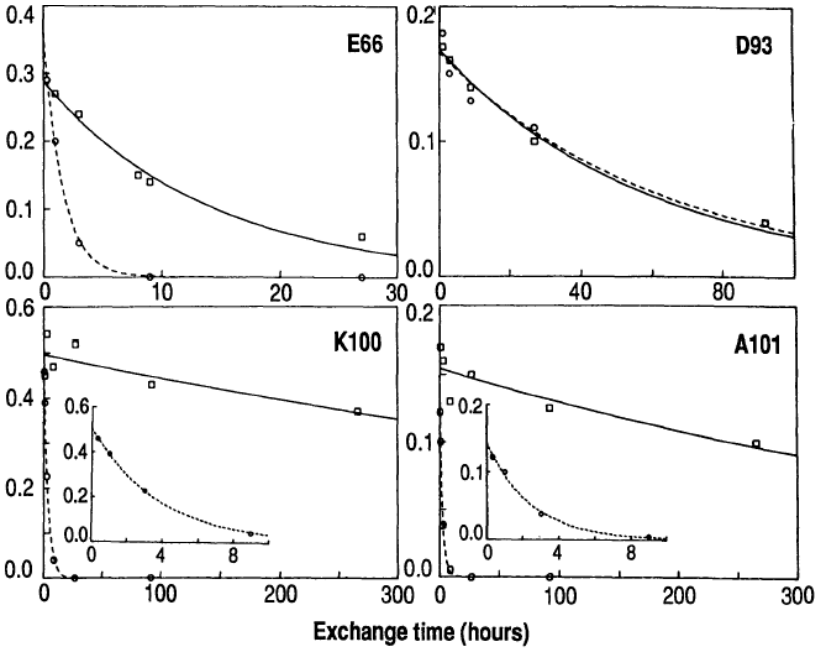


Red residues – changes in dynamics and chemical shift
Green residues – no changes in dynamics and chemical shifts
Blue residues – changes in dynamics and chemical shift, but don't interact with peptide

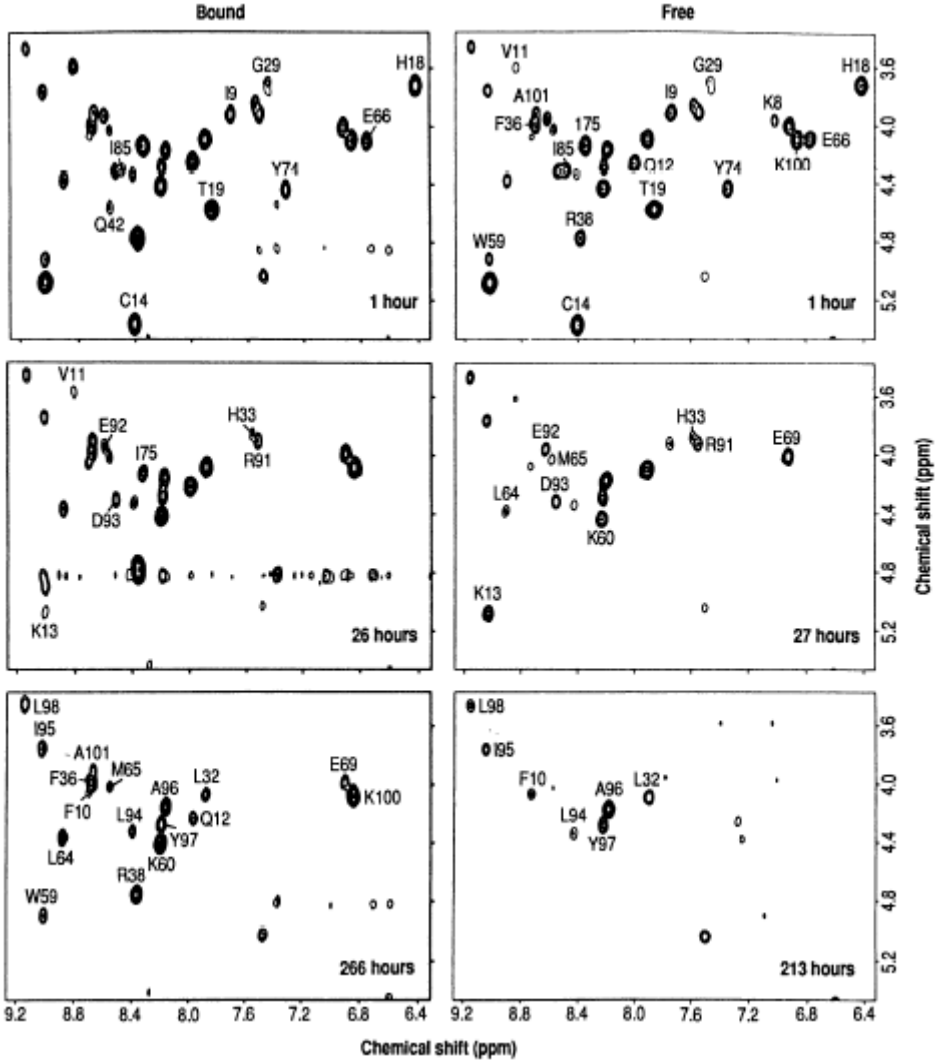
NMR Analysis of Protein-Ligand Interactions

Protein Deuterium Exchange Changes Upon Ligand Binding

- Presence of Ligand "Protects" NHs from solvent
  - ♦ results in a slower NH exchange rate for NHs in ligand binding site



Antibody binding site on Cytochrome C



Science, New Series, Vol. 249, No. 4970 (Aug. 17, 1990), 755-759.

NMR Techniques for Determining the Structure of a Ligand in a Complex

To determine the structure of a bound ligand using NMR methods, we must be able to **assign the signals belonging to the ligand within the complex and determine structural constraints belonging to those signals (ie. NOEs).** To determine the structure of a ligand bound to a receptor, we must be able to distinguish the ligand signals from receptor signals:

spectrum of ligand **spectrum of receptor** **spectrum of complex**

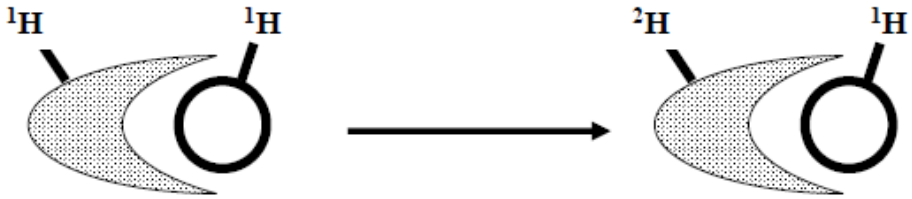
Note: not necessarily a superposition of ligand and receptor spectra

How to separate signals of receptor from those of ligand within the complex?? **To distinguish ligand signals from receptor signals, the following techniques are available:**

- **DEUTERATION**
- **ISOTOPE LABEL/FILTER**
- **TRANSFER NOE**

Deuteration of Receptor

replacement of the protons by deuterons in the receptor will eliminate the signals of the receptor within the complex:



deuteration of receptor

spectrum of complex

spectrum of ligand bound to deuterated receptor

Deuteration of the receptor would normally be used to study the structure of a ligand in a complex under conditions of slow exchange. **This method has largely been superseded by isotope filtering methods because:**

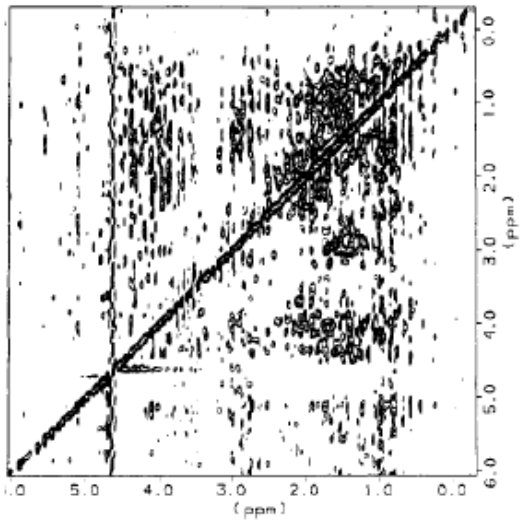
- ✓ expense of deuteration
- ✓ ability to edit spectra of complexes for the ligand signals or receptor signals in isotope filtering experiments
- ✓ exchangeable protons on receptor (ie. amide protons) are not removed from spectra collected in water

Example: Use of Deuteration to Determine the Structure of a Peptide within a Peptide-Protein Complex

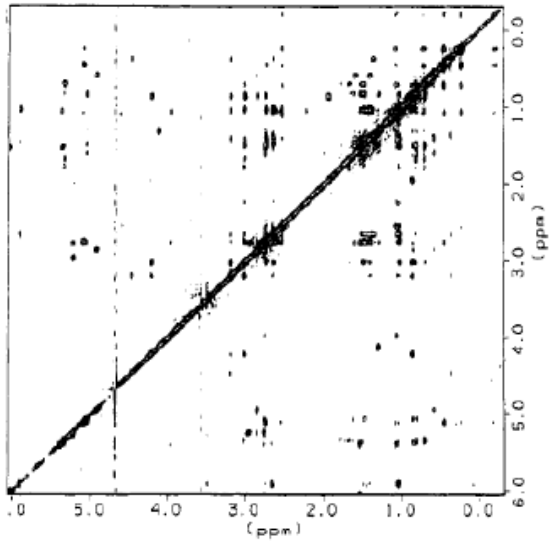
complex: Cyclosporin A (CsA, cyclic 11-mer peptide) + cyclophilin (CyP, 17.7 kDa)

binding: $K_D \sim 10^{-8}$ M

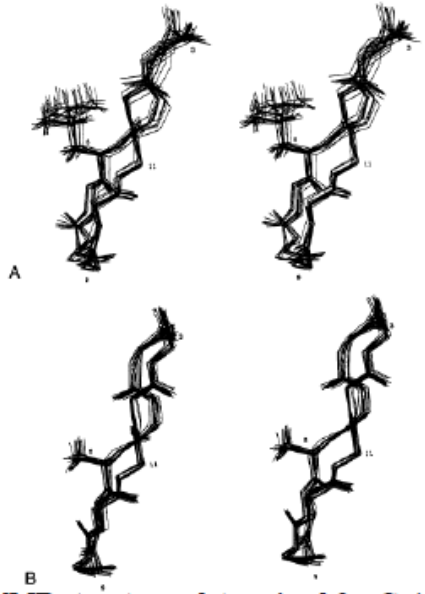
sample and methods:
perdeuterated CyP by recombinant expression in bacteria grown in deuterated algal hydrolysate in D2O
NMR sample consisted of 0.4mM complex with excess CyP
2D homonuclear experiments run (COSY,TOCSY,NOESY)
66 intraresidue and 55 interresidue NOEs obtained and used in XPLOR (simulated annealing protocol)



Portion of the NOESY spectrum of CyP-CsA complex (175-ms mixing time, 20 mM phosphate and 250 mM NaCl, pH 6.8, 25 °C). Complex formed with protonated CyP, 0.75 mM complex.



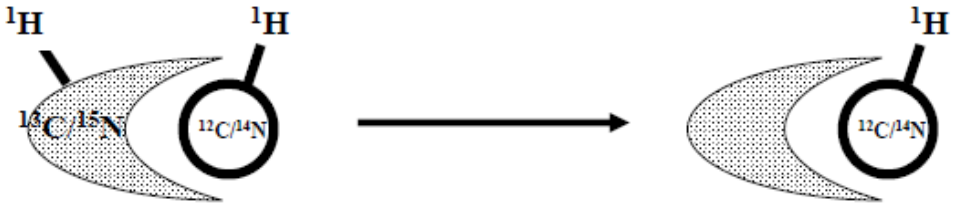
Portion of the NOESY spectrum of CyP-CsA complex (175-ms mixing time, 20 mM phosphate and 250 mM NaCl, pH 6.8, 25 °C). Complex formed with fully deuterated CyP, 0.4 mM complex.



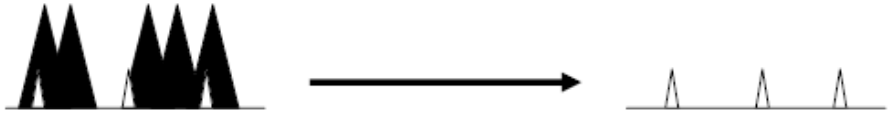
Stereoview of the NMR structures determined for CsA and MeAla6-CsA bound to deuterated CyP. Only the backbone atoms and the side chain of residue 6 are shown. (A) Superposition of the 16 best energy-minimized structures of CsA. (B) Superposition of the 17 best energy-minimized structures of MeAla6-CsA.

Isotope Filtering

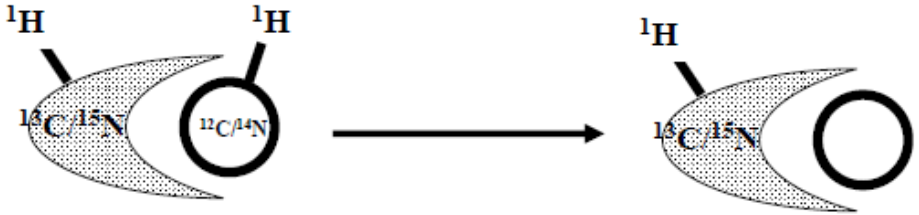
labeling of the receptor (or ligand) with $^{13}C/^{15}N$ permits the use of an "**isotope filtering**" nmr experiment to select for signals in the spectrum from protons that are either bonded to $^{13}C/^{15}N$ or $^{12}C/^{14}N$:



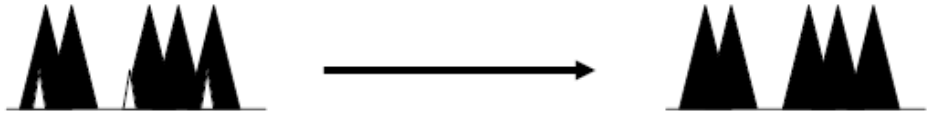
isotope filter to select for $^{12}C/^{14}N$



spectrum of complex **spectrum of ligand**



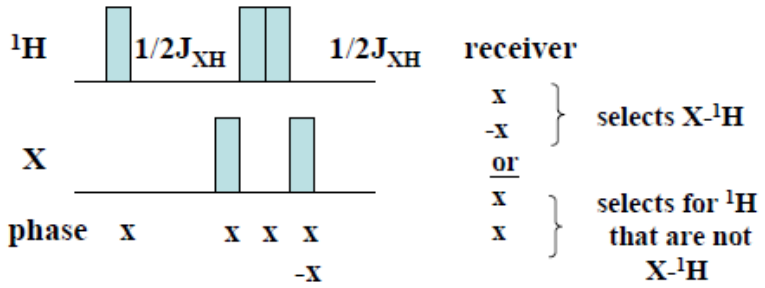
isotope filter to select for $^{13}C/^{15}N$



spectrum of complex **spectrum of receptor**

The Isotope Filter

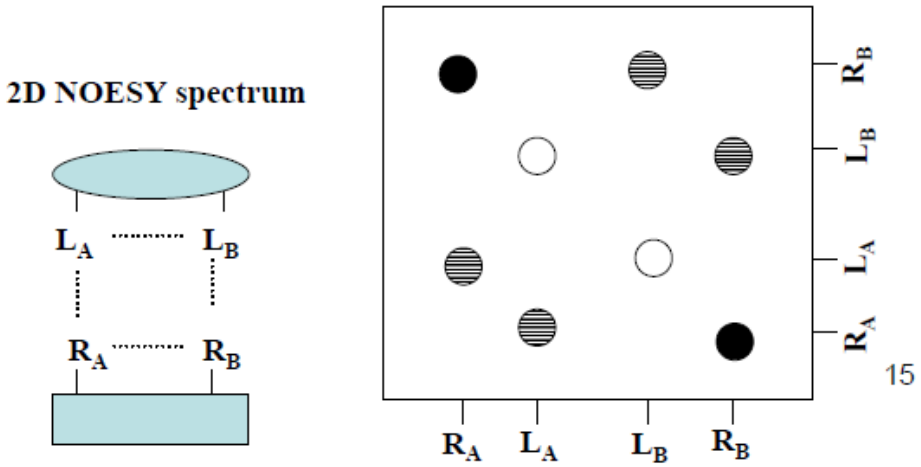
In an isotope filtered experiment, a 90° pulse is replaced by:



If the receptor (R) or ligand (L) is isotopically labeled, an isotope filter at each end of a NOESY can be use to select for:

- ✓ NOEs between protons on the ligand (○)
- ✓ NOEs between protons on the receptor (●)
- ✓ NOEs between ligand protons and receptor protons(◍)

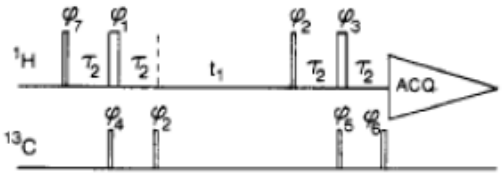
depending on the phase cycling scheme used. Isotope filtering usually used to study the structure of a ligand in a complex under conditions of *slow exchange* (transfer NOE used under conditions of *fast exchange*).



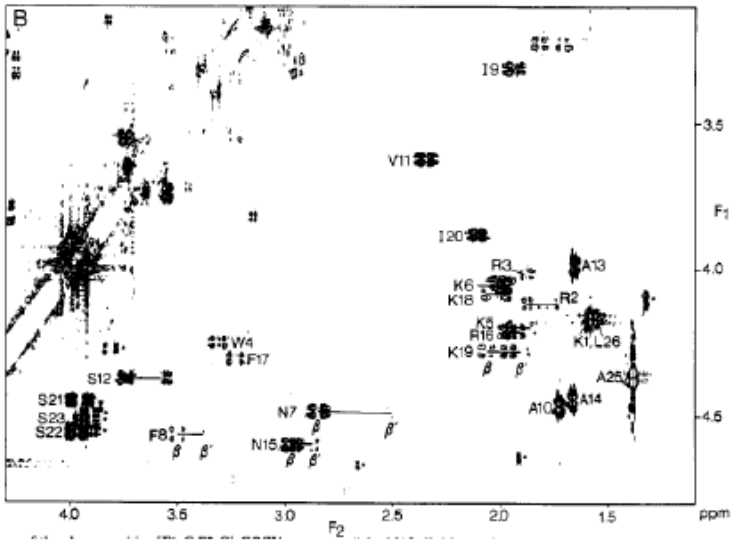
Example: Use of Isotope Filter to Determine the Structure of a Peptide Within a Complex

Use Isotope Filter to Assign Unlabeled Peptide Within Complex:

- Protein is ^{13}C and ^{15}N labeled
- Ligand is unlabeled
- Observe COSY or NOE cross peaks for unlabeled ligand in presence of labeled protein
  - Filtered – observe ^{1}H attached to ^{12}C or ^{14}N



^{12}C-filtered COSY



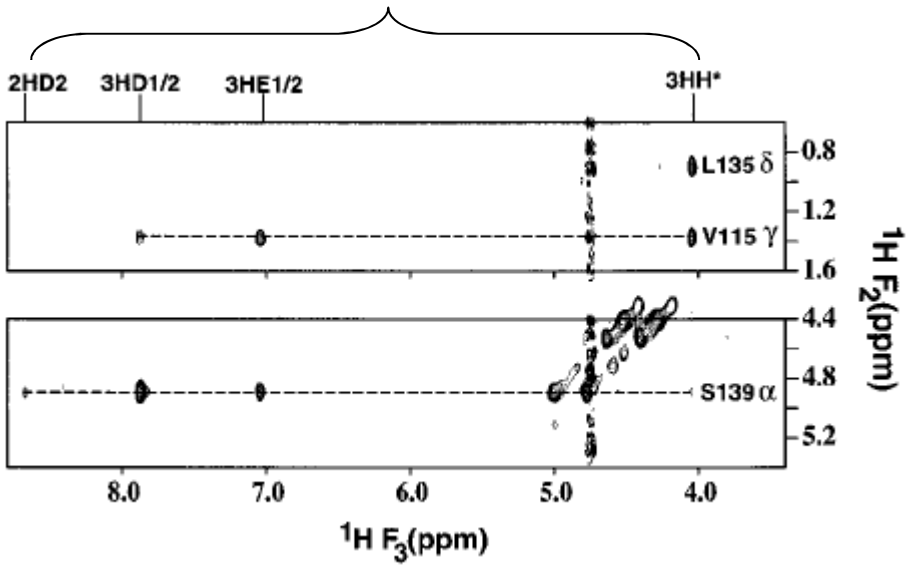
Aliphatic Region of the phase-sensitive [F1-C,F2-C]-COSY spectrum of the M13-CaM complex recorded in D20 solution. Cross peaks marked by asterisks (*) originate from a slight excess of free M13 peptide.

NMR Analysis of Protein-Ligand Interactions

Protein-Ligand Complexes Using Multi-Dimensional NMR

- Protein is ^{13}C and ^{15}N labeled
- Ligand is unlabeled
- Observe NOEs between Protein and Ligand using combined edited & filtered NMR experiments
  - Edited – observe ^{1}H attached to ^{13}C or ^{15}N
  - Filtered – observe ^{1}H attached to ^{12}C or ^{14}N

NOE crosspeaks to $^{1}H,^{12}C$ coupled pairs from ligand



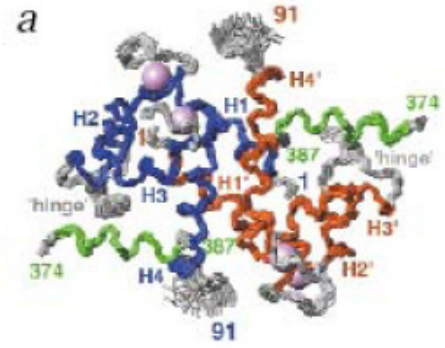
Diagonal peaks correspond to $^{1}H,^{13}C$ coupled pairs from protein

Example: Use of Isotope Filter to Determine the Structure of a Peptide-Protein Complex

Use Isotope Filter to Assign Intermolecular NOES Within Complex:

- Protein is ^{13}C and ^{15}N labeled
- Ligand is unlabeled
- Observe NOEs between Protein and Ligand using combined edited & filtered NMR experiments
  - Edited – observe ^{1}H attached to ^{13}C or ^{15}N
  - Filtered – observe ^{1}H attached to ^{12}C or ^{14}N

NMR data from the negative regulatory domain of p53 (unlabeled) bound to ^{13}C,^{15}N labeled S100B(ββ). *a*, Strip extracted from the 2D ^{12}C filtered TOCSY spectrum to illustrate proton assignments for Thr 377, Arg 379, and Leu 383 of the p53 peptide. *b*, A strip from a plane of the 3D ^{13}C edited, ^{12}C filtered NOESY experiment illustrating intermolecular NOE correlations from Met 79ε of S100B(ββ) to several residues of the p53 peptide.

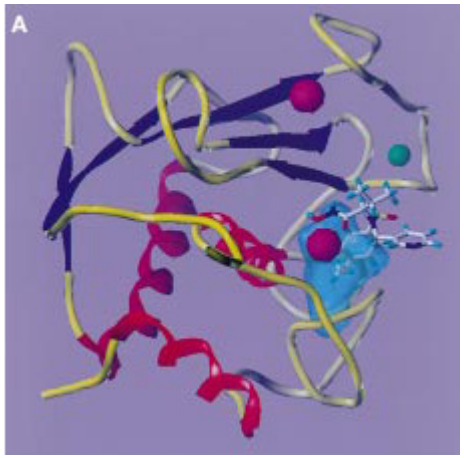


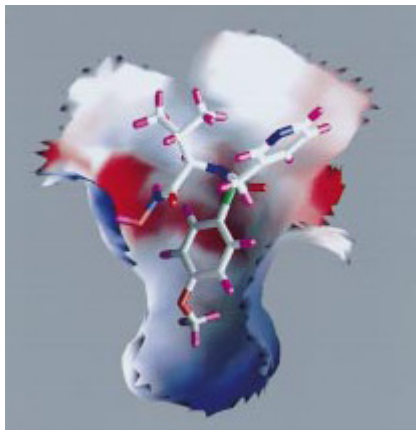
Backbone superposition of a family of 40 NMR derived structures of the negative regulatory domain of p53 in complex with Ca^{2+} loaded S100B(ββ). The helices of the S100β subunits are blue (H1-H4) and red (H1′-H4′), Ca^{2+} ions are magenta, and the negative regulatory domain of p53 (residues Gly 374– Thr 387) are green. Loops are gray including loop 2, which is labeled as the 'hinge' on both subunits.

NMR Analysis of Protein-Ligand Interactions

Protein-Ligand Complexes Using Multi-Dimensional NMR

- Protein-Ligand NOEs are added to all other restraints used to calculate the protein structure

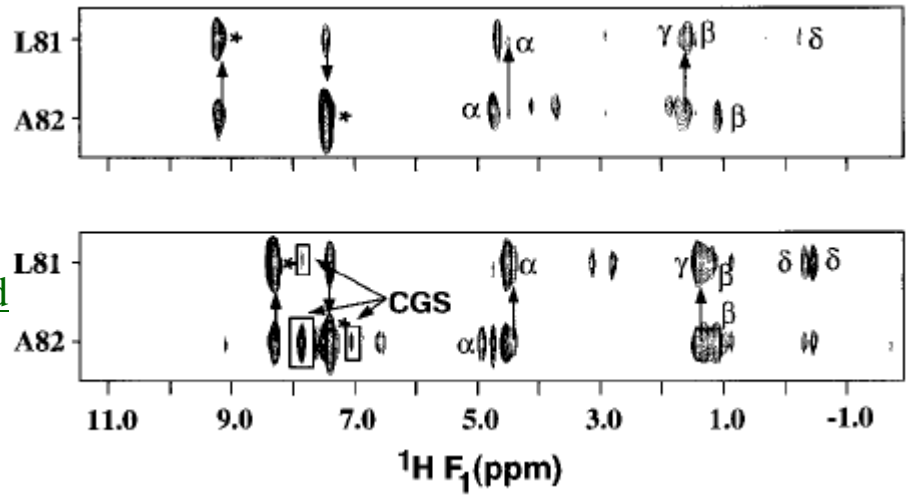




3D ^{15}N-edited NOESY

Free Protein

Protein-Ligand Complex

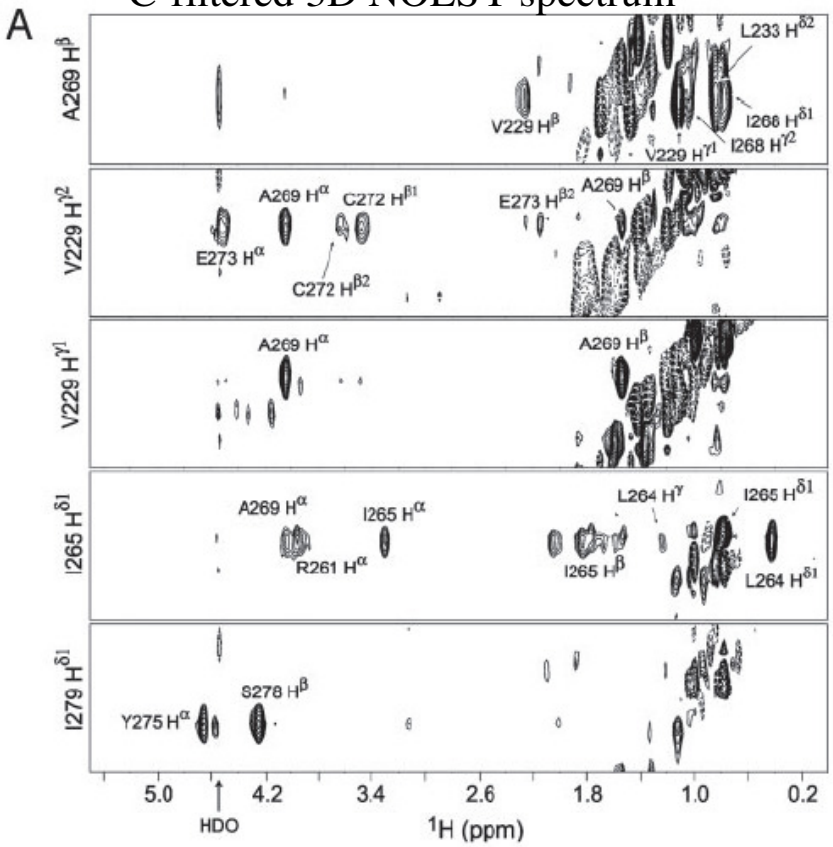


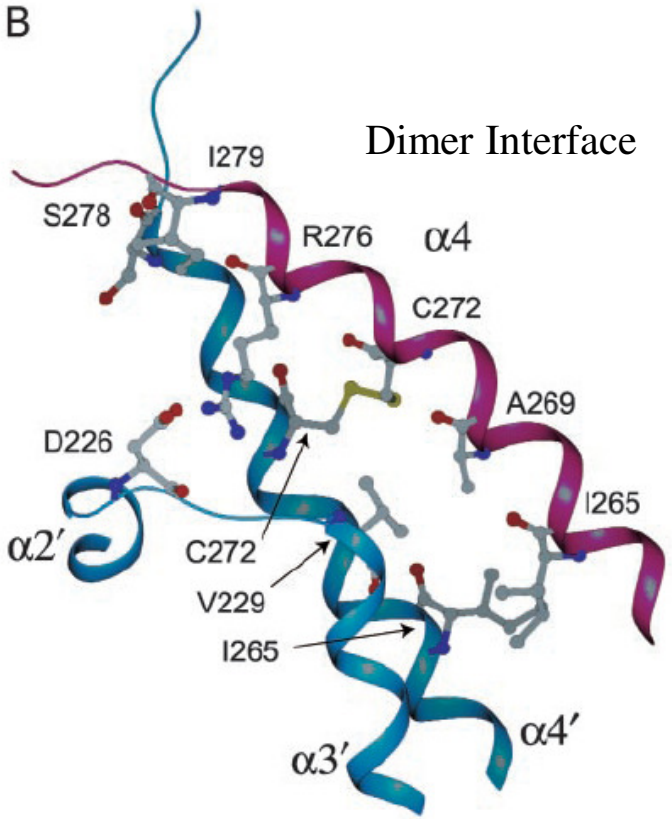
NMR Analysis of Protein-Ligand Interactions

Similar Approach Can Be Used For Larger Protein-Protein Complexes

- For a homodimer, mix labeled and unlabeled samples of the protein
  - 50% of the dimer would contain one unlabeled and one labeled monomer
  - 25% of the dimer would contain both labeled monomers
  - 25% of the dimer would contain both unlabeled monomers

Intermolecular NOEs from ^{13}C-edited ^{12}C-filtered 3D NOESY spectrum



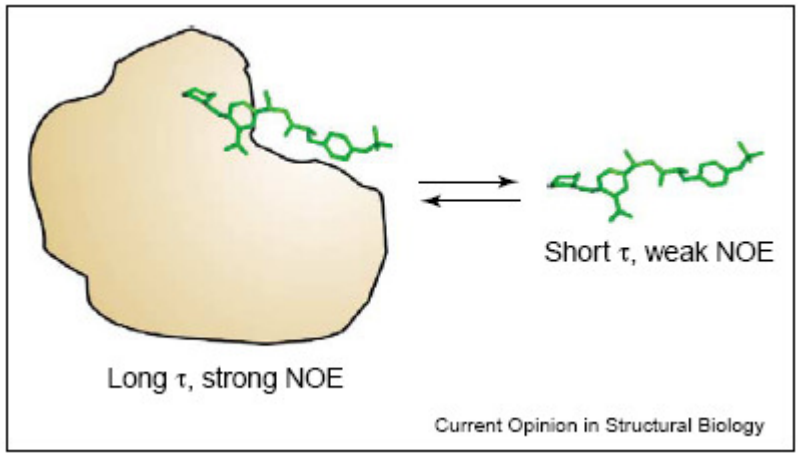


PNAS **2004** 101 (6) **1479–1484**

NMR Analysis of Protein-Ligand Interactions

Protein-Ligand Complexes From Transfer NOEs

- Applied to Systems Under Fast exchange
  - To observe a transfer NOE:
    - $K_D > 10^{-7}$ M
    - $k_{off} > T_1^{-1}$
    - collect a standard 2D ^{1}H NOESY experiment
  - Ligands show a single set of resonances averaged over bound and free forms
  - Ligand is 10-50 fold excess relative to protein
  - A strong NOE developed in the complex is ***transferred*** to the free ligand state and measured from the free ligand resonances
    - applicable to large MW complexes
  - Observed NOEs can be used to determine a bound conformation for the ligand
  - Change in the ***Sign*** of the NOE crosspeak relative to the diagonal



Current Opinion in Structural Biology 2003, 13:581–588

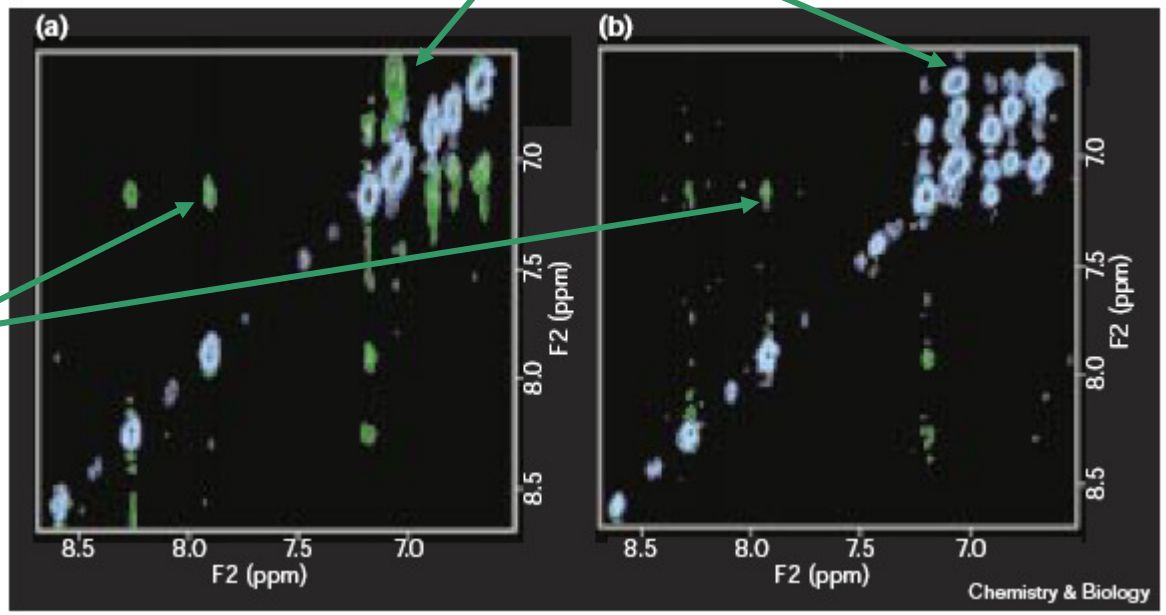
NMR Analysis of Protein-Ligand Interactions

Protein-Ligand Complexes From Transfer NOEs

Change in sign of cross peak indicates binding

2D NOESY spectra
Positive peaks –cyan
Negative peaks - green

No change in sign, no binding



Free Ligands

Ligands + Protein

Chemistry & Biology 1999, Vol 6 No 10

NMR Analysis of Protein-Ligand Interactions

Protein-Ligand Complexes From Transfer NOEs

- A docked peptide-protein complex based on transfer NOEs