Part II: NMR Applications
NMR to monitor ligand binding

\[ k_{\text{ex}} = k_{\text{on}}[L] + k_{\text{off}} \]

\[ \Delta \omega = \omega_f - \omega_b \]
Binding of nucleotide to protein

Dose dependent resonance shifts can be fit to obtain Kd
Fraction bound of labeled protein

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

\(\overline{\omega}\) : observed chemical shift
Shifts may be color coded onto surface to identify ligand binding site

Caveats?
Monitoring Protein/Protein Interactions by HSQC

(a) 50 µM Rbx2 + 108 µM Ube2M
(b) 50 µM Rbx2 + 94 µM Ube2F
ILV labeling

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

Isoleucine
Leucine
Valine

(add alpha-ketoacid precursors to ILV 30 minutes prior to induction)
$^{13}$C-$^1$H HSQC of ILV labeled protein
Measuring pKa by NMR

(pH 4.5 - 9.5)

pKa of 7.2, elevated for Glu
pH dependence disappears in E152Q mutant

(pH 4.5 - 9.5)

I199

I136

I136
Preview: folding, dynamics and catalysis
Timescales of Protein Dynamics

From Henzler-Wildman and Kern, Nature 2007
Fast Dynamics

Nuclear Spies Report Dynamics

Amide Nitrogen

Amide Hydrogen
Tranverse Relaxation Effects
Resonance Linewidth

\[ \frac{1}{\pi T_2} = (d_{CH})^2 \tau_c \]

Rate constant: \( R_2 = 1/T_2 \)
Transverse Relaxation

Ensemble of Nuclear Spins

Random Phase
No NMR Signal

Phase Synchronization
NMR Signal!

Loss of NMR Signal
$1/T_2$
A Major Source of Relaxation is Brownian Rotational Diffusion

\[ \tau_m : \text{rotational correlation time} - \text{the time to rotate through one radian} \]
The spin echo to measure $R_2$

Resonance intensity weighted by $\exp(-R_2 2\tau)$
Spin Echo Spectra at Variable $\tau$ Delay

$\text{Re } S(\nu)$

$\tau = 0$ ms

$\tau = 20$ ms

$\tau = 40$ ms
Extracting R2 from Spin-Echo Data

\[ I(\tau) = \exp(-R_2 \tau) \]

This can be thought of as a type of 2D NMR Experiment
Backbone Dynamics of Calmodulin Studied by $^{15}$N Relaxation Using Inverse
L-Selected Two-Dimensional NMR Spectroscopy: The Central Helix Is Flexible

Gaetano Barbato, Mitsuhiko Ikura, Lewis E. Kay, Richard W. Pastor, and Ad Bax

FIGURE 2: Contour plots of the most crowded region of the $^1$H-$^{15}$N shift correlation spectrum recorded with the $T_2$ scheme of Figure 1b, using relaxation delays, $T$, equal to (a) 8.7 ms, (b) 43 ms, and (c) 156 ms.

FIGURE 3: (A) $T_1$ and (B) $T_2$ decay curves for four amino acids with substantially different relaxation rates. Drawn curves represent calculated least-squares best fits to single exponential decays.
Relaxation of populations

Before 180 @EQ

After 180
The Inversion Recovery Experiment to measure R1

Note lack of CS evolution during delay
Inversion Recovery Data

Figure 4.31  Experimental results from an inversion-recovery sequence. The group of peaks at lower field (i.e. to the left) all have slightly longer $T_1$'s than those of the high-field group.
Analysis of Inversion Recovery Data

\[ M_z = M_{z_{eq}} (1 - 2e^{-tR_1}) \]
Backbone Dynamics of Calmodulin Studied by $^{15}$N Relaxation Using Inverse L-Selected Two-Dimensional NMR Spectroscopy: The Central Helix Is Flexible

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Figure 3: (A) $T_1$ and (B) $T_2$ decay curves for four amino acids with substantially different relaxation rates. Drawn curves represent calculated least-squares best fits to single exponential decays.
The “Frequency” Dependence of Relaxation Rates, R1 example

Efficient relaxation if $1/\tau_c = \omega$
Relaxation Rates Depend on Amplitude and Frequency of Local Field Fluctuations

\[ R_1 (N) = c^2 J(\omega) \]

Square of fluctuating local field

Spectral Density Function

\[ J(\omega) = \frac{\tau_m}{1 + (\omega \tau_m)^2} \]
$^{15}\text{N} - ^{1}\text{H}$ spin pair has four states
Spectral Density Functions

\[
R_1 = \frac{d^2}{4} \left[ J(\omega_H - \omega_N) + 3J(\omega_N) + 6J(\omega_H + \omega_N) \right] + c^2 J(\omega_N)
\]

\[
R_2 = \frac{d^2}{8} \left[ 4J(0) + J(\omega_H + \omega_N) + 3J(\omega_N) + 6J(\omega_H) + 6J(\omega_H - \omega_N) \right]
\]
\[
+ \frac{c^2}{6} \left[ 3J(\omega_N) + 4J(0) \right]
\]

where \(d = \left( \frac{\mu_0 h\gamma_N \gamma_H}{8\pi^2} \right) \left\langle \frac{1}{r_{NH}^3} \right\rangle\) \(c = \Delta \left( \frac{\omega_N}{\sqrt{3}} \right)\)

Farrow et.al, (1995) J. Biomol. NMR 6, 153
Rigid amide groups
Detecting mobile amide groups
R1 and R2 are not uniform
Model Free formalism accounts for internal motions

Lipari-Szabo (Model Free)

\[ J(\omega) = \frac{2}{5} \left[ \frac{S^2 \tau_m}{(1 + \omega^2 \tau_m^2)} + \frac{(1 - S^2)\tau_e}{(1 + \omega^2 \tau_e^2)} \right] \]

where

\[ \frac{1}{\tau} = \frac{1}{\tau_e} + \frac{1}{\tau_m} \]

"Rotational" Brownian Motion
Heteronuclear NOE measurements

- Measure saturated and unsaturated experiments and take the intensity ratio for each peak

Farrow and Kay, Biochemistry, 1993
The heteronuclear NOE

\[
\begin{align*}
M_N &\propto (N_{\alpha\alpha} - N_{\beta\alpha}) + (N_{\alpha\beta} - N_{\beta\beta}) \\
M_H &\propto (N_{\alpha\alpha} - N_{\alpha\beta}) + (N_{\beta\alpha} - N_{\beta\beta})
\end{align*}
\]

Saturation equalizes $\beta\beta$ and $\beta\alpha$, $\alpha\beta$ and $\alpha\alpha \Rightarrow M_H = 0$

$R_1$ transitions are an independent return to equilibrium
The heteronuclear NOE

\[ M_N \propto (N_{\alpha\alpha} - N_{\beta\alpha}) + (N_{\alpha\beta} - N_{\beta\beta}) \]

\[ \text{NOE} = \frac{I(\text{sat}) - I(\text{unsat})}{I(\text{unsat})} = 1 + \left( \frac{\gamma_H}{\gamma_N} \right) d^2 \left\{ 6J(\omega_N + \omega_H) - J(\omega_N - \omega_H) \right\} / R_1(N) \]
Flexible

Rigid

$h\text{NOE and Dcp2}$

$^{15}\text{N-}^1\text{H}} \text{ NOE}$

Floor and Gross, unpub.
hNOE versus structure

Low NOE (dynamic)  High NOE (rigid)
R1, R2 and NH-NOE: three relaxation rates

-> three fit parameters: $\tau_m, \tau_e, S^2$
Timescales of Protein Dynamics

From Henzler-Wildman and Kern, Nature 2007
Spectral Manifestations of Exchange

\[ k_{\text{ex}} = k_f + k_r \]

\[ \Delta \omega = \omega_a - \omega_b \]

\[ \omega_a \]
\[ \omega_b \]

Slow

\[ k_{\text{ex}} < \Delta \omega \]

Intermediate

\[ k_{\text{ex}} \sim \Delta \omega \]

Fast

\[ k_{\text{ex}} > \Delta \omega \]
Methionine Specific Labeling

Inhibition of KSHV Pr stabilizes the dimeric conformation

Slow interconversion between monomer and dimer

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

$^{15}$N TYR HSQC
GB1-γDCP2-NB

7 out of 8 resonances detected
Slow Exchange Reported by Unnatural Amino Acid

Lampe et al, JACS 2008
Monitoring unfolded states by NMR

Unstructured regions fluctuate from fast (ns-ps) to slow ms-us timescales

Gross et al, Cell 2003
Paper Discussion

Solution Structure and Dynamics of Ras p21·GDP Determined by Heteronuclear Three- and Four-Dimensional NMR Spectroscopy† ‡

Per J. Kralis,§‖ Peter J. Domaille,⊥ Sharon L. Campbell-Burk,⊥,# Thomas Van Aken,⊥ and Ernest D. Laue*§

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Received October 19, 1993; Revised Manuscript Received December 21, 1993*
Determining Structures by NMR

\[ \text{NOE} = f(\gamma_e) \cdot \frac{1}{r^6} \]

We can see distances that are \( \leq 5 \, \text{Å} \).
A Real 2D NOE Experiment of a Small Peptide

A projection through both dimensions gives a 1D spectrum

- $H_N^\text{-}H_{\text{aliph}}$ crosspeaks
- $H_N^\text{-}H_a$ crosspeaks
- $H_N^\text{-}H_N$ crosspeaks
Interpretation of 2D NMR Spectra

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

- The intensity of the crosspeak often quantifies the interaction.

- A heteronuclear experiment (1H-15N) would not have diagonal crosspeaks.
Higher Dimensionality 3 and 4D Heteronuclear Experiments on Isotopically Labeled (15N-13C) Proteins

2D NOESY of a 76 residue protein homodimer (effectively 18kD) in D$_2$O

In practice, even small proteins have very crowded 2D spectra making assignment very difficult. In this case the fact that it is in D$_2$O simplifies the spectra because the amide protons exchange for deuterium and are not visible.
Benefit of C13 and N15 labeling of Proteins for NMR

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

2D nOe Expt. on unlabeled protein

3D nOe Expt. on N15-labeled protein

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

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

nOes between Protons Attached to 13C and Protons and Attached to 13C
Side-chain protein assignments

H(CCO)NH \ i - 1 \text{ res.}

All Carbon’s H’s at i-1 to N-H pair.

15N-TOCSY \ i \text{ res.}

All H’s at i to N-H pair.
Close interatomic distances in secondary structures

- Alpha-helix
- Parallel beta-sheet
- Antiparallel beta-sheet
- Type I turn
- Type II turn
H$^\alpha$ chemical shifts and secondary structure

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

- H$^\alpha$ chemical shifts in $\alpha$-helices are on average 0.39 ppm below “random coil” values, while $\beta$-sheet values are 0.37 ppm above random coil values.

Wishart, Sykes & Richards

Chemical shift index (CSI)

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

<table>
<thead>
<tr>
<th>exp’tl $H^\alpha$ shift rel. to reference</th>
<th>assigned CSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>within ± 0.1 ppm</td>
<td>0</td>
</tr>
<tr>
<td>&gt;0.1 ppm lower</td>
<td>-1</td>
</tr>
<tr>
<td>&gt;0.1 ppm higher</td>
<td>+1</td>
</tr>
</tbody>
</table>

Chemical shift indices

- any “dense” grouping of four or more “-1’s”, uninterrupted by “1’s” is assigned as a helix, while any “dense” grouping of three or more “1’s”, uninterrupted by “-1’s”, is assigned as a sheet.
- a “dense” grouping means at least 70% nonzero CSI’s.
- other one regions are assigned as “coil”
- **this simple technique assigns 2ndary structure w/90-95% accuracy**
- similar useful relationships exist for $^{13}C_{\alpha}$, $^{13}C=O$ shifts
• NMR provides information about structure
  • chemical shifts <=> local electronic environment
  • coupling constants <=> torsion angles
  • NOE, ROE <=> interproton distances
  • residual dipolar couplings <=> bond orientation

• and dynamics
  • relaxation times
  • NOE, ROE

• Most of the data describe
  • local environment of the protons
  • relative to each other
  • not the global conformation of the molecule
• **Distance**
  NOE: The distance between i and j is a function of the NOE intensity $D_{ij} \sim C(\text{NOE}_{ij})^{-6}$

  H-bonds: Identified by slowly exchanging amide $H_N$ protons

• **Angles**
  Side Chain $\chi$ and backbone torsion identified from J-coupling experiments

  Chemical Shift also gives Angular Information

• **Residual Dipolar Couplings**
  Bond Orientations Relative to an Alignment Tensor
• Molecular Dynamics with Simulated Annealing starting from random coordinates

• Goal is to minimize the hybrid energy function

- Additional Unambiguous Experimental Restraints
- E-ForceField
- E-NOEs
- E-Angles
- E-H_bonds
- E-Chemical_shift
- E-Dipolar_couplings
The hybrid energy function

- Structure calculation = minimization of hybrid energy function (target function) which combines
  1. different experimental data
  2. \textit{a priori} information (force field)

\[
E_{hybrid} = \sum_l w_l E_l = w_{\text{bond}} E_{\text{bond}} + w_{\text{angle}} E_{\text{angle}} + w_{\text{improper}} E_{\text{improper}} + w_{\text{nonbonded}} E_{\text{nonbonded}} + w_{\text{unambig}} E_{\text{unambig}} + w_{\text{ambig}} E_{\text{ambig}} + \ldots + w_{\text{torsion}} E_{\text{torsion}} + w_{J\text{coup}} E_{J\text{coup}} + w_{RDC} E_{RDC} + \ldots
\]
Minimization by molecular dynamics

- MD solves Newton’s eqns. of motion:

\[
\frac{d^2 \mathbf{r}_i}{dt^2} = -\frac{c}{m_i \mathbf{r}_i^2} E_{\text{hybrid}}
\]

- Molecular dynamics can overcome local energy barriers

- Temperature control and variation: minimization by simulated annealing
• Key problem is ambiguity in NOE assignments

• Need for higher dimensional data: 3D & 4D

• Need for heteronuclear data

• Need for better calculational strategies that can deal with ambiguous data
Errors in data: error bounds

- Cumulative error in $D_{ij}$ is treated by using loose error bounds $L \ldots U$

- Precise value not (too) critical:
  loose bounds restrict conformational space

- However, consequences for:
  - precision of structure
Figure 11. Illustration of the progressive improvement in the precision and accuracy of NMR structure determinations obtained by increasing the number of experimental restraints. (A) Best superposition of the backbone atoms of 8 restrained molecular dynamics structures of phoratoxin (46 residues) calculated on the basis of 331 interproton distance restraints. (B) Best-fit superposition of the backbone and representative side chains of 11 simulated annealing structures of BDS (43 residues) calculated on the basis of 409 interproton distance restraints (with no stereospecific assignments) together with distance restraints for 12 hydrogen bonds and 23 φ backbone torsion-angle restraints derived from 3JHH coupling constants. (C) Best-fit superposition of the backbone and representative side chains of 42 simulated annealing structures of BDS calculated on the basis of 489 interproton distance restraints (including stereospecific assignments for 19 out of 30 β-methylene groups) supplemented by distance restraints for 12 hydrogen bonds and 23 φ and 21 χ; side-chain torsion-angle restraints derived from a qualitative analysis of the intraresidue NOE and coupling constant data. (D) Best-fit superposition of the backbone atoms and representative side chains of the 41 simulated annealing structures of the C terminal domain of cellulohydrolase 1 (46 residues) determined on the basis of 554 interproton distance restraints (including stereospecific assignments for 17 out of 22 β-methylene groups), restraints for 12 hydrogen bonds, and 33 φ, 24 ψ, and 25 χ; torsion-angle restraints obtained using a data base search procedure on the basis of NOE and coupling constant data. The structures in A, B, C, and D correspond to what can be termed first-, second-, third-, and fourth-generation NMR structures, respectively. In all these examples, the interproton distance restraints have only been classified into three broad ranges: 2.7 Å, 3.3 Å, and 5.0 Å, corresponding to strong, medium, and weak NOEs, respectively.
Paper Discussion

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P21 RAS(1-166) GDP structure determined by NMR

Ensemble of structures: backbone RMSD in Switch 2

Poorly Defined Loop
Loop containing critical residues for catalysis poorly defined
Disorder from lack of restraints or mobility?
What does T2 tell us about the Switch 2 loop containing Q61?
Is the heteronuclear NOE consistent with fast ps-ns motions in active site?

Think about T1 versus $\tau_m$: how does T1 change if $\tau_m$ is reduced?
T1/T2

Related to $\tau_m$ for each residue
T1