Protein assignment strategies in the solid-state
Sample preparation

Types of sample:
- Microcrystalline
- Fibrillar
- Membrane
- Other

Considerations:
- Linewidth
- Salt concentration
- Hydration
- Temperature

Changes in Antamanide spectra as a function of sample preparation
Acquisition conditions

Temperature

- Ensure heating from spinning/rf pulses adequately compensated
- Optimal dynamics for sensitivity/resolution

Spinning speed

- Volume of rotor (can you get 15mg’s sample into it)
- Avoid rotational resonance conditions
- Sample heating

Decoupling

- Higher decoupling fields typically give best results (heating)
- Ensure no interference with spinning, pulse sequences applied to low-γ nuclei
- Can you manage without?
Overview

- Identify spin-systems
- Correlate spin-systems with amino acids
- Identify sequential connectivities
Identification of spin systems

Identify all connected $^{13}$C atoms

- PDSD/DARR
- Double quantum expt’s
  - SQ/SQ
  - DQ/SQ
- Considerations
  - Efficiency
  - Adjacency (multiple bond transfers)
Single Quantum/Single Quantum

Range of expts:

- Proton driven spin diffusion
- Dipolar assisted rotary resonance
- RFDR/C7/MELODRA MA etc ....
- J-coupled transfers TOBSY

TOBSY spectrum of antamanide ($\omega_r=20$ kHz)
Double quantum recoupling experiments

- Sequence of transfer encoded in sign of cross-peak
- Efficiency of transfers high
- Can filter diagonal

However

- Application at high fields difficult

2D DREAM spectrum of antamanide ($\omega_r=20$ kHz)
DQ/SQ Correlation Spectra

- Different chemical shift dispersion to SQ/SQ expts.
- Maybe helps resolve ambiguities

DQ/SQ Spectrum of Crh (10kDa) acquired at 600 MHz
Double Quantum recoupling achieved with SPC-5 (C7 variant that works at higher spinning speeds), $\omega_r = 11$ kHz
Identification of amino acids

- Connectivity's
  - Work through side chain resonance
- Chemical shifts
  - Identify characteristic chemical shift patterns for given amino acids
  - For random-coil shifts of amino acids see additional handout.
Assignment of the individual amino acids

- Can now assign peaks to individual classes of amino acids

- Side chains enable us to link Cα/CO resonances to amino acid types

- How do we work out which order they are in?
Nitrogen the link

- Nitrogen can be correlated to $i$ and $i-1$ residues
- Enables us to make assignments sequential

How do we assign the nitrogen spectrum?
Assigning nitrogen spectra - CP

\[
\begin{align*}
\text{Assigning nitrogen spectra - CP} \\
\text{Diagram showing chemical structure and \((\pi/2)_y\) spin lock and decoupling for } ^1H, ^13C, \text{ and } ^15N.
\end{align*}
\]
Assigning nitrogen spectra - CP

Diagram showing chemical structures and spin lock decoupling with angles $\pi/2$ and $\pi$. The diagram includes molecules labeled with subscripts and Greek letters.
Nitrogen/Carbon Correlation

- Magnetisation transfers in both directions
- Can assign nitrogen resonances through $^{13}$C shifts

(a)

![Diagram showing nitrogen/carbon correlation](image)
Directed transfers – making CP specific

1) Hartmann-Hahn condition
   small for $^{15}\text{N}/^{13}\text{C}$ correlations
2) Application of weak rf fields
   for cross-polarisation

Advantages

- Sensitivity (magnetization transferred in one direction)
- Alleviates problems with broad-banded transfers
Directed transfers – specific transfers

\[ \frac{\pi}{2} y \] Spin Lock

\[ ^1H \]

Decouple

Decouple

Set carrier to CO or C\(\alpha\) freq.

\[ ^{13}C \]

\[ ^{15}N \]
NCA expt ($^{15}$N to C$_{\alpha}^i$)

NCA Expt.

- Good spectral dispersion in C$_{\alpha}$

750 MHz, $\omega_r=12$ kHz, $^{13}$C/$^{15}$N cross polarization $B_1^{15N}=30$ kHz; $B_1^{13N}=15$ kHz (centred on 45 ppm)
NCO expt (15N to COi-1)

NCO Expt.

- Poor spectral resolution in CO

750MHz, $\omega_c=12$ kHz, $^{13}$C/$^{15}$N cross polarization $B_1^{15N}=30$ kHz: $B_1^{13N}=15$ kHz (centred on 180 ppm)
Spectral crowding in the CO region

Problem

- Poor resolution in the carbonyl region, particularly acute in $\alpha$-helical proteins

Solution:

- Make assignments relying solely on $C_\alpha^i$ and $C_\alpha^{i-1}$
- Transfer magnetization from N$\rightarrow$CO$\rightarrow$C$\alpha$
$\text{N} \rightarrow \text{CO} \rightarrow \text{C}_\alpha : \text{A solid state N(CO)CA}$
Sidechains already assigned

Identified spin systems which we can correlate to particular amino acids

We know Cα/CO shifts for given amino acid type

TOBSY spectrum of antamanide (ωr=20 kHz)
N(CO)CA - Antamanide

600 MHz, $\omega_r=20$ kHz, $^{13}$C/$^{15}$N cross polarization $B_{1}^{15N}=30$ kHz: $B_{1}^{13N}=50$ kHz (centred on 180 ppm)
Directionality of transfer determined by direction of sweep of rf field in NC cross polarisation

- Walk through assignment
- $^{15}$N degeneracy?
Implementation of the N(CO)CA expt.

Considerations:

- Transfer efficiency (CP, $^{13}\text{C}/^{13}\text{C}$ mixing)
- Sensitivity – optimize transfers
  - N$\rightarrow$C transfer
    - Double cross-polarization
    - TEDOR (hardware dependent)
  - C$\rightarrow$C Homonuclear recoupling
    - Directed/Coherent transfers RR-tickling (80% efficiency)
    - PDSD/DARR – weak correlations to side chains
Assignment of protons

- Resolution in proton spectra under MAS poor
- Homonuclear decoupling methods for acquiring proton spectra in direct dimension exist but resolution poor
- Typically proton chemical shifts obtained through correlation with carbon/nitrogen
- Implemented as a HETCOR experiment, with homonuclear decoupling in the indirect dimension
1H/X Correlation Spectroscopy

- Spin lock typically shift to prevent spin-diffusion
- Spinning speeds typically low to aid LG decoupling
Lee-Goldberg Decoupling

- Protons irradiated off-resonance by $\omega_1/\sqrt{2}$
- Precess at magic-angle, averaging homo-nuclear interactions
- Scaling other interactions e.g. chemical shift
- Typically implemented as a phase ramp (as opposed to frequency jump)
1H/X Correlation Spectroscopy

- Resolution, primarily from low-γ nuclei

- $^1$H Spectra more sensitive to changes in environment
  - H-bonding
  - Ring current effects

1H/15N HETCOR Spectrum of SH3
750 MHz, $\omega_r=8$ kHz, 750μs cross-polarization
So what is stopping us!

We currently rely on:

- Unambiguous assignment of $^{15}$N spectrum (no overlap)
- Ability to resolve all resonances in the $\text{C}_{\alpha}/\text{CO}$ region

- Identify spin systems
- Correlate them to particular amino acids
- Link them together sequentially
Target proteins

- Some proteins more amenable than others!

- To date many of the larger proteins assigned by solid-state NMR have been β-sheet structures (convenient for amyloid based studies)

- What about all the α-helical proteins (membrane proteins)

Spera & Bax 1991 JACS
Challenging systems

• Higher fields
  – As inhomogeneous broadening is no longer the problem, improvements in resolution are realised at higher field

• Better decoupling (J-decoupling)
  – Refocus J couplings during evolution periods

• Progress to 3D/4D experiments
15N resolved 13C/13C spectra
3D correlation spectra of DsbB

Li et al. 2007 Protein Science 17: 199-204
DsbB, 4 transmembrane domain (20kDa), data acquired at 750 MHz.
Transfers made using CP and DARR. Decoupling 75 kHz TPPM, 90kHz CW during CP.
Summary

Progress critically dependent on

• Sensitivity !!!!!
• Resolution (sample, magnetic field)
• Chemical shift dispersion
• Careful optimization of transfer steps
• Sample stability

However

• Tools for assignment exist
Use of assignments

• Local electrostatic environment
  – Hydrogen bonding
  – Ring current effects

• Conformation
  – Backbone chemical shifts (torsion angles)

• Subsequent structural studies
  – Structural studies (direct determination of distances/torsion angles etc).

• Next seminar
Structures from solid-state NMR: Oriented samples
Oriented samples

Necessary to introduce macroscopic alignment:
1) Crystallization
2) Oriented membranes
3) Fibres (Silk/DNA)
Orientation constraints from multiply labelled proteins

For proteins and peptides

- Need resolution
- Characterise backbone orientation

Solution

- Exploit $^{15}$N chemical shielding anisotropy
- $^1$H-$^{15}$N dipolar coupling
- Characterise orientation of peptide plane
PISEMA spectra

Polarization inversion spin exchange at the magic angle
- $^{15}$N chemical shielding anisotropy
- $^{15}$N-$^1$H dipolar interaction

Good scaling factor (0.82) and can be implemented in 3/4D experiments to improve resolution

$$\left(\frac{\pi}{2}\right)_X$$

$^1\text{H}$

- $Y$
- $Y + \text{LG}$
- $Y - \text{LG}$

Decouple

$X$

- $X$
- $X$
- $X$
- $-X$
PISEMA spectra of Fd coat protein
Tilt of helices from PISA wheels

PISA
Polarity Index Slant Angle

Position of wheels in PISEMA spectra give orientation of helices in samples

Amphipathic helix on bilayer surface

TMD 30° with respect to bilayer
Assignment of PISEMA spectra

- Position of PISA wheel gives helix tilt
- Amino acid selective labelling provides assignment
- Assignment gives rotation axis of peptide

PISEMA Spectra of amino acid selectively labelled Fd cost protein (Marrassi, 2002)
Extracting structure: dipolar waves

Dipolar waves
- dipolar coupling versus residue
- periodicity arises from repeating structure (e.g. α-helix)
- enables comparisons to be made with rdc’s in solution
- disruption in ideal nature of secondary structure readily apparent
Dipolar waves: Fd coat protein

Breaks in wave indicate:

- Start of new secondary structure
- Deformation in secondary structure (kinks in helices)
Structures from solid-state NMR: Magic angle spinning
Structures from MAS data

- **Assignment**
  - Spin systems
  - Sequential assignment

- **Chemical Shifts**
  - Secondary shifts
  - Dihedral angle prediction

- **Structural Constraints**
  - Proton driven spin diffusion
  - Torsion angle measurements
  - Specific distance constraints
Conformation dependent shift

• $C_\alpha$, CO and $C_\beta$ chemical shifts are sensitive to backbone conformation
Secondary chemical shifts

Can compare chemical shifts with random coil to extract structure

\[ \Delta \delta = \delta_{c\alpha} - \delta_{c\beta} = \{\delta_{c\alpha}(\text{obs}) - \delta_{c\alpha}(\text{rc})\} - \{\delta_{c\beta}(\text{obs}) - \delta_{c\beta}(\text{rc})\} \]

- \( \Delta \delta > 0 \) \( \rightarrow \) \( \alpha \) helical conformation
- \( \Delta \delta < 0 \) \( \rightarrow \) \( \beta \) sheet conformation

Typically \( \beta \) sheet resonances are more negative than \( \alpha \) helical resonances are positive

Convenient does not require assignment of all CO’s

Luca, S. et al. J. Biomol. NMR 20: 325-331
Secondary chemical shifts

Quick check on secondary structure e.g. analysis of Het-S prion

Chemical shifts as structural constraints

- Comparing the observed assignments with a database of structures whose assignment is known allows the backbone torsion angles to be determined.

- Several programs now available:
  - TALOS (http://spin.niddk.nih.gov/NMRPipe/talos/)
  - DANGLE (part of ccpn)

- Typically give range of dihedral angles consistent with chemical shifts.

- Can be used as restraints in structure refinement.
Distance constraints in proteins

- $^{13}$C-$^{13}$C spectra show high number of correlations
- Transfer dipolar in origin – through space interaction
- Should have a number of through space constraints analogous to a 2D NOE experiment

The problems

- Dipolar truncation effects
- Long distances and weak couplings between $^{13}$C atoms

Dipolar truncation effects

Structurally interesting long range distances lost

Weak coupling to remote spin

Strong coupling between adjacent $^{13}$C atoms

Magnetization trapped
Partial and selective labelling

- Partial and selective labelling
  - $1,3^{-13}$C Glycerol
  - $2^{-13}$C Glycerol
- Introduces $^{13}$C at only selective sites
- Reduces strong couplings
- Increases resolution
  - Less peaks
  - Fewer J-couplings
- Prevents relayed transfers

Partial and selective labelling

[1,3-13C]-Glycerol
[2-13C]-Glycerol

NB/ Care with fractional labelling different isoptomers present, not just a percentage incorporation at a given site

Partial and selective labelling of SH3

[2-$^{13}$C]-Glycerol                                                    [1,3-$^{13}$C]-Glycerol

500 ms proton driven spin diffusion data on SH3 (Castellani, F. et al. 2002 Nature 420:98-102)
Long range couplings observed
Distances from PDSD data

- Measure pdsd at 50, 100, 200 and 500 ms mixing time
- Calibrate on conformation independent distances
- Cross peaks grouped into four categories (in all cases lower limit 2.5Å)
  - 2.5→3.8Å peaks between sequential Cα’ s appear in first 50ms
  - ~4.6Å (sequential Cα’ s and Cβ’ s) and 4.6→5.4Å interstrand Cα’ s and Cα’ s) within first 100ms
  - ~5.8Å (sequential Cβ’ s)
  - All other interactions <7.5Å
- Grouping used as a basis for structure calculations
Structure of SH₃

Is partial labelling necessary

- Interactions between $^1$H and low $\gamma$ nuclei remain
- Transfer driven by:
  - heteronuclear coupling between $^1$H and low $\gamma$ nuclei
  - $^1$H-$^1$H homonuclear interactions not averaged by MAS
Build-up of cross peak intensity

Increasing the distances probed

- $^{13}\text{C}/^{13}\text{C}$ interactions – limited by strength of dipolar couplings
- $^1\text{H}$ spectra difficult to manipulate
- Acquire low-$\gamma$ resolved $^1\text{H}/^1\text{H}$ spectra
CHHC Spectra

- Long range distances (red)
- Good agreement between distance and cross peak intensities

Gardiennet et al. 2008 J. Biomol. NMR 40:239-250
Summary

Assignment
- Spin systems
- Sequential assignment

Chemical Shifts
- Secondary shifts
- Dihedral angle prediction

Structural Constraints
- Proton driven spin diffusion
- Long distance constraints (CHHC)
Best results to date!

Backbone rmsd 0.4Å
Heavy atom rmsd 1.0Å

Wasmer et al. 2008 Science 319:1523-1526
References

1. Spin Dynamics: Basics of Nuclear Magnetic Resonance, Malcolm Levitt
2. Biomolecular NMR, Jeremy Evans
3. Principles of Magnetic Resonance, C.P. Slichter

Proton driven spin diffusion
Appendix 1

\[ C_1 = \frac{\sqrt{2}}{3} \omega_0 \delta_\sigma \sin \beta \cos \beta [3 + \eta \cos 2\alpha] \]

\[ S_1 = \frac{\sqrt{2}}{3} \omega_0 \delta_\sigma \sin \beta \eta \sin 2\alpha \]

\[ C_2 = \frac{\omega_0 \delta_\sigma}{3} \left( \frac{3}{2} \sin^2 \beta - \frac{\eta}{2} (1 + \cos^2 \beta \cos 2\alpha) \right) \]

\[ S_2 = \frac{\omega_0 \delta_\sigma}{3} \eta \cos \beta \sin 2\alpha \]