Experiment and Modelling in Structural NMR

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3D modern techniques

[03002]

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SiO₂ structure from NMR studies

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The aim of the school is to provide basic understanding on the coupling of theoretical Modelling and Simulation methods to the Experiments in structural NMR, for biology and material science. It is composed of Seminars in the morning and Practical Training in the afternoon involving both the theoretical and experimental aspects.

A limited number of scholarships can be obtained for PhD students
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3D NMR experiments applied to proteins

Essential constituents of all living organisms

Linear chains consisting of 20 L-amino acids

Their folding to particular 3D structures triggers their biological function

Attractive targets for therapeutic applications once their structure-activity relationship is understood
NMR studies of peptides (<50aa)

**General considerations**
- Chemically synthesized molecules (SPPS)
- Isotopic labelling expensive → homonuclear $^1$H-$^1$H 2D experiments

**Spectral assignment**
- Starting point for magnetisation transfer: backbone NH protons (experiments in H$_2$O)
- Spin system identification via scalar couplings: 2D COSY, RELAYS, TOCSY
- Sequential assignment: 2D NOESY

**Extraction of geometrical informations**
- Dihedral angle $\phi$ determination from $^3$J$_{HN-HN}$ couplings: 2D-COSY-DQF (Karplus equations)
- Interproton distance determination: 2D NOESY (build up with several $\tau_m$)

NMR studies of small proteins (50-100aa)

**General considerations**
- Favorable $T_2$ relaxation
- Increased $^1$H signal density → increased spectral complexity and peak overlapping (structuration dependent)

**Spectral assignment and structure elucidation**
- Same strategy as previously
- Addition of one more dimension to increase resolution: first 3D experiment NOESY-TOCSY, Nature 332, 374 (1988)

**Breakthrough**
- Recombinant protein expression → affordable $^{15}$N labelling (natural abundance 0.366%)
  - The gene encoding the protein of interest is inserted to the plasmids
  - Plasmids contain genes confering resistance to antibiotics (selection)
  - Plasmids are inserted to the bacteria (transformation)
  - Use of rich media (LB) for cell replication
  - Induction in poor media (M9) containing $^{15}$NH$_4$Cl
  - Rare codons
  - Protein folding and inclusion bodies
  - Post-translational modifications

03002-p.3
Heteronuclear 3D experiments
- Resolve the $^1$HN overlapping by the use of $^{15}$NH frequency: 4ppm versus 30-35ppm

Sensitivity considerations (S/N)

$$S \sim n \ A \ (1/T) \ B_0 \ \gamma_{obs}^{3/2} \ \gamma_{exc}^{3/2} \ T_2 \ \sqrt{N_{scan}}$$

n: sample quantity (v*c) A: active nuclei abundance

Cryoprobe technology: N↓

NOESY(TOCSY) - fHSQC

TOCSY mixing: use clean CSL schemes to suppress dipolar cross relaxation and NOESY/ROESY artifacts

Going from 2D to 3D

2D NOESY ($\tau_m$=150ms)
4 scans

3D NOESY-HSQC H-H-N ($\tau_m$=150ms)
8 scans
3D data analysis

Heteronuclear correlation building blocks

HMQC (J₁H, J₁5N, T₁, T₂)

HSQC (J₁H, J₁5N, T₁, T₂)

refocused HSQC (J₁5N, T₂)

Axial peak suppression: φ₁ and rec cycled x₁, -x₁

Quadrature detection at the second dimension using φ₁ incrementation:

TPPI (axial peak at the edges, folding)
States (axial peak in the middle, aliasing)
States-TPPI (axial peak at the edges, aliasing)
Heteronuclear correlation building blocks

**HMOC (J_\(1^H, J_{1^H \rightarrow 1^H}, T_{1^H}, T_{2^H})**

**Refocused HSQC (J_{1^H \rightarrow 1^H}, T_{1^H}, T_{2^H})**

**HSQC (J_{1^H \rightarrow 15N}, T_{1^H}, T_{2^H})**

**Sensitivity enhanced HSQC (J_{1^H \rightarrow 15N}, T_{1^H}, T_{2^H})**

SUMMATION → 2y
DIFFERENCE → 2x
Increase S/N : √2 (neglecting relaxation)

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NMR studies of medium size proteins (100-200aa)

- Due to increased spectral complexity, peak assignment is difficult through 3D H-H-N TOCSY/NOESY-HSQC experiments

- Slow molecular tumbling results in rapid T2 proton relaxation: COSY and TOCSY HN → Hα magnetisation transfer fails especially in helical regions (\(^3J_{\text{HN-Hα}} \approx 3\text{Hz}\))
NMR studies of medium size proteins (100-200aa)

- Recombinant expression of $^{13}$C, $^{15}$N labelled proteins using $^{15}$NH$_4$Cl and $^{13}$C$_6$-glucose → access to triple-resonance experiments

- Resolve the $^1$HC overlapping by using $^{12}$CH frequency: 5ppm versus 65-70ppm for the aliphatics

- Use of heteronuclear couplings $^1$H-$^{15}$N, $^1$H-$^{13}$C, $^{13}$C-$^{13}$C, $^{15}$N-$^{13}$C ($^1$J and $^2$J) for unambiguous intra- and inter-residual assignment

- Magnetisation transfers implicating slowly relaxing nuclei

Backbone assignment via *out and back* 3D experiments

- HNCO (100)
- HNCA (50/15)
- HN(CA)CO (13/4)
- HN(CO)CA (71)
- HNCACB (4-1.7/1.3/0.5)
- HN(CO)CACB (13-9)
The HNCA experiment

Water magnetization at z prior to WATERGATE module
Proton decoupled version (longer T2 relaxation for 15N, 13C nuclei)
Constant time at 15N dimension
1^J_CaCβ couplings not refocused during t_b
Δ1 = 1/41^J_HN = 2.7m
Δ2 = 12m (relaxation optimized, 1/4 J_NCa ~ 22m)
Same sequence for HNCO experiment (via Ca-CO channel interconversion)
Experimental aspects of triple resonance experiments

- Shaped pulses
- Pulsed field gradients
- Water suppression

Hard pulses

Excitation profile of a rectangular 90° pulse $\tau_p = 1$ ms

Rectangular hard pulses (polychromatic): $\gamma B_1 / 2\pi \gg \Delta F$

Typical hard pulse lengths @ 800MHz, max admitted power:

$^1$H $\rightarrow$ 7–9μs

$^{15}$N $\rightarrow$ 39.5μs

$^{13}$C $\rightarrow$ 14μs
Shaped selective pulses

Amplitude modulation: first trials

Rectangle
Triangle
Sinc

Amplitude modulation: peak selection

Gaussian_1

90° Gaussian δ and J evolution during pulse:
Shaped selective pulses

Amplitude/phase modulation: band selection (top-hat profile)
Gaussian Cascade and BURP families

Gaussian Cascade family
- Universal pulses: Q5 (90°, 6.18) and Q3 (180°, 3.41/3.448)
- Excitation pulse: G4 (7.82)
- Inversion pulse: G3 (3.556)

BURP families
- Universal pulses: U-BURP (90°, 4.666) and RE-BURP (180°, 4.64/5.814)
- Excitation pulses: E-BURP1 (4.514) and E-BURP2 (4.952)
- Inversion pulses: I-BURP1 (4.498) and I-BURP2 (4.53)

Figure of merit (depends on the shape): \[ \text{FM} = \tau_p \Delta \Omega \]
Frequency shifted (laminar) pulses: \[ \Delta \phi = 2\pi \tau_p \Delta F / k \]
Examples of selective excitation

Advantages
• zoom into a precise spectral region
• suppression of indesired signals (solvent)
• speed-up experiments
• resolution increase
  - precise measurement of δ et J
  - refocusing of homonuclear couplings
• possibility to direct transfers
• possibility to treat an homonuclear system as heteronuclear

Examples of 2D selective experiments

classical NOESY

semi-soft NOESY

soft NOESY
Selective excitation in triple resonance experiments

- Differentiation between $^{13}$C$_{\alpha}$ (or $^{13}$Caliph) and $^{13}$CO regions
  Use of rectangular pulses: $\tau_{180} = \sqrt{3}/2\Delta\Omega$ and $\tau_{90} = \sqrt{15}/4\Delta\Omega$
  where $\Delta\Omega$ is the difference of pulse carrier and the first null point
  Problems: inhomogenous excitation/inversion and cryoprobe arcing if executed simultaneously high power $^{13}$C and $^{15}$N pulses
  Use of universal top-hat pulses (or interleaved G4 and G4tr)

90° pulse comparison: 36µs square and 300µs G4

ATTENTION
Offset-dependent transient Bloch-Siegert phase shifts in homorefocusing

180° pulse comparison: 33µs square and 200µs G3

- Selective perturbation of $^1$HN region (BEST experiments)
- Solvent selection (flipback or suppression)

Broadband inversion and decoupling

- High field spectrometers → large $^{13}$C spectral width in Hz → imperfect inversion/decoupling
  → use of adiabatic pulses: smoothed CHIRP and WURST families

180° pulse comparison: 28µs square and 500µs CA-WURST-4

Adiabatic decoupling:
Use P4M5 supercycle
Use bi-level decoupling for reduction of sidebands in $^1$H-$^1$H-$^{13}$C and $^1$H-$^1$H-$^{15}$N experiments
Homonuclear decoupling → offset dependent Bloch-Siegert frequency shifts

- Adiabatic $^{15}$N inversion and decoupling is used to reduce power and thus probe heating, important in rapid scan conditions
- Adiabatic pulses are tolerant to Rf inhomogeneity
- Interesting pseudoadiabatic pulses: Bip and power-BIBOP for inversion and power-BEBOP for excitation
Pulsed field gradients

Linear variation of $B_0$ at a certain direction
- Coherence transfer pathway selection: $\sum \gamma_i p_i G_i = 0$
- Artifact suppression per scan ie $^1H^12C$ artifacts with short phase cycling (...subtraction)
- Solvent suppression and control of water magnetisation (radiation damping)
- Translational diffusion measurements
- Magnetic Resonance Imaging

Gradient sensitivity enhanced HSQC ($J_{SS}, T_{1H}, T_{2S}$)

Water suppression

Strong water (~50M) signal deteriorates spectral dynamic range
High fields + cryoprobes $\rightarrow$ strong radiation damping $\rightarrow$ large water signal

Strategies
- Observe an heteroatom
- Presaturation: forbidden in the case of fastly exchangable protons
- Coherence selection with gradients
- Return of water at z axis: polynomial sequences (jump and return...), water selective flip-back pulses, manipulation of radiation dumping
- Dynamic filters: based on the differences in relaxation rates or diffusion coefficients
- Selective defocusing of water via a gradient echo (WATERGATE, excitation sculpting)
- Purging pulses: $B_0$ or Rf gradient
The HN(CA)CO experiment

INEPT version

\[ \Delta_1 = \frac{1}{4} J_{HN} = 2.7 \text{m} \]
\[ \Delta_2 = 12 \text{m} \text{ (relaxation optimized, } \frac{1}{4} J_{NCO} = 22 \text{m}) \]
\[ \Delta_3 = 3.5-4 \text{m} \text{ (relaxation optimized, } \frac{1}{4} J_{OC_{\alpha\alpha}} = 4.5 \text{m}) \]

The HN(CO)CA experiment

mixed INEPT / DQ version

\[ \Delta_1 = \frac{1}{4} J_{HN} = 2.7 \text{m} \]
\[ \Delta_2 = 13.5 \text{m} \text{ (relaxation optimized, } \frac{1}{4} J_{NCO} = 33 \text{m}) \]
\[ \Delta_3 = 8 \text{m} \text{ (relaxation optimized, } \frac{1}{2} J_{OC_{\alpha\alpha}} = 9 \text{m}) \]
The HNCACB experiment

The HNCACB experiment:

- Mixed INEPT / RELAY sequence
  - $\Delta_1 = 1/4J_{HN} = 2.7 m$
  - $\Delta_2 = 12 m$ (relaxation optimized, $1/4J_{NC} \sim 22 m$)
  - $\Delta_3 = 3.6 m$ (relaxation optimized, $1/4J_{JC} = 7.1 m$)
  - $1J_{JC}$ and $1J_{JC}$ couplings not refocused during $t_2$
  - $C_\alpha$, $C_\beta$ peaks of opposite sign

Rapid pulsing - BEST experiments

- In $^1H$-$^15N$ correlation experiments $^1HN$ longitudinal dipolar relaxation is accelerated if the rest of the protein protons (aliphatics) and $^1H_2O$ are unperturbed (at z axis)
- Substitution of $^1H$ hard pulses with shaped ones (top-hat).

SOFAST-HMQC ($J_{SS}$, $T_2$, $T_2S$) and BEST-HSQC ($J_{SS}$, $T_1$, $T_2S$)

- $1/4J$ hard pulses
- PC9 pulse (flip-angle 120°, Ernst angle)
- Band-selective excitation pulse
- Band-selective universal 180° pulse

- $1/4J$ hard pulses
- PC9 pulse (flip-angle 90°)
- Band-selective universal 180° pulse
Rapid pulsing - BEST experiments

SOFAST-HMQC

BEST-HSQC

90° pulse → 1770µs Q5.1000

180° pulse → 1260µs Q3.1000

1H carrier frequency @ 7.999ppm

P5M4 15N decoupling using 2ms CA-WURST-2 adiabatic pulse

1scan, 128 transients, AQ=70ms, RD=300ms, Total Experimental Time: 55s

Fast out and back experiments: BEST-HNCA

1H: 180° broadband inversion pulse → 155µs power-BIBOP pulse
10kHz inversion, tolerant to 20% inhomogeneity
Conclusions on BEST *out and back* experiments

- Usefull for protonated proteins < 150aa (above, fractional deuteration is required)

- Other fast methods are based on reduced sampling at the indirect dimensions:
  
  Spectrum compression through folding

  Exponential sampling and maximum entropy

  Projection reconstruction (radial sampling)

  Hadamard transform

  Filter Diagonalisation Method

  Sharc NMR

...
Backbone and side chain assignment via transfer 3D experiments

In H₂O:

In D₂O:

The HCCH-TOCSY experiment

Refocused INEPT / z-TOCSY

Constant time at both indirect dimensions

$\Delta_1 = 1/2 J_{HC} = 37m$ (optimized for CH₂)

Purging of residual water magnetization with B₀ and RF gradients
NMR studies of large proteins (>200aa)

- Decrease transverse $^{13}$CH relaxation rates through deuteration:
  Expression in D$_2$O → up to 70% deuteration (isotopic effect)
  Expression in D$_2$O+U[13C, D]-glucose → up to ~100% deuteration
  Use of pyruvate or α-ketobutyrate/α-ketoisovalerate for production of methyl protonated perdeuterated proteins.
  *(back-exchange of ND to NH, introduction of D decoupling)*

- Decrease transverse $^{15}$N$^1$H relaxation rates through TROSY effect at high field:
  Selection of the slower relaxing component due to the destructive interference of cross-correlated relaxation (DD - CSA)

- At high fields transverse $^{13}$CO relaxation rates increase due to CSA
  Use i-HNCA instead of HN(CO)CA