Deuteration: Structural Studies of Larger Proteins

• Problems with larger proteins
• Impact of deuteration on relaxation rates
• Approaches to structure determination
• Practical aspects of producing deuterated proteins

Tendamistat 8 kDa

Cdc42 21 kDa
HR1b
9 kDa
$\tau_c = 6\,\text{nsec}$

Cdc42/ACK
26 kDa
$\tau_c = 18\,\text{nsec}$
Effects of Increasing Molecular Size

• number of resonances
  - crowded spectra

• faster relaxation
  - broad lines
  - low intensity
  - overlap
  - long experiment time
Transverse relaxation times as a function of protein size

\[ C_x \rightarrow C_y N \quad \Delta = 20-24 \text{ msec} \]

\[ C_x \rightarrow C_y \quad \Delta = 20-27 \text{ msec} \]

but \( T_2 \) often less than 30 msec
Linewidth vs. Correlation Time

Sensitivity $\alpha$:

$\Pi_n \sin(\pi J\Delta)$  
active

$\Pi_m \cos(\pi J\Delta)$  
passive

$\exp(-R_2 \Sigma\Delta)$

Linewidth $\Delta \nu_{1/2} = 1/\pi T_2$

<table>
<thead>
<tr>
<th>$\tau_c$</th>
<th>MW</th>
<th>5ns</th>
<th>10ns</th>
<th>15ns</th>
<th>25ns</th>
</tr>
</thead>
<tbody>
<tr>
<td>10kDa</td>
<td>10kDa</td>
<td>20kDa</td>
<td>30 kDa</td>
<td>50kDa</td>
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</tr>
</tbody>
</table>

Graphs showing variations in linewidth with different correlation times and molecular weights.
Relaxation Mechanisms

- Dipole-Dipole interactions (DD)
- Chemical shift anisotropy

$$R_{1,2}^{\text{dip}} (X) \sim D \cdot l(l+1) \cdot (\gamma_x \gamma_y)^2 \cdot \sum_i J(\omega_i)$$

**internal DD contributions**

$$X = ^{13}\text{C}, ^{15}\text{N}$$

$$\gamma_D / \gamma_H = 1 / 6.5$$

$$d_{CD} / d_{CH} = 1 / 16$$
Impact of deuteration on relaxation rates

\[ \tau_c = 12 \text{ nsec} \]

![Graph showing relaxation rate constant/Hz for Cα, N, Hα, HN with different colored bars representing removed and fixed by sidechain deuteration.]

- White bars: removed by sidechain deuteration
- Black bars: fixed
Impact of deuteration on relaxation rates

\[ \tau_c = 18 \text{ nsec} \]

**T2**

- \( N \)
- \( H_N \)
- \( C_{\alpha}(H) \)

**T1**

- \( N \)
- \( C_{\alpha}(H) \)
- \( H_N \)
- \( H_\alpha \)
Effects of deuteration on Cα relaxation times

Isolated C-H pair

- $T_2(\text{CD})/T_2(\text{CH})$
- $T_1(\text{CD})/T_1(\text{CH})$

Effects of deuteration on Cα relaxation times

- Isolated C-H pair
- J(0) dominate
- $J(\omega_C-\omega_D)$ effects
Approaches to the Structure Determination of Larger Proteins

- For $\tau_c$ up to ~12 ns (20 kDa) - $^{13}$C/$^{15}$N-labelling
- For $\tau_c$ up to ~18 ns (35 kDa) - fractional deuteration and $^{13}$C/$^{15}$N-labelling
- For $\tau_c$ above ~18 ns - selective protonation and $^{13}$C/$^{15}$N-labelling
Triple Resonance NMR and Random Fractional Deuteration

- Backbone assignments
- Side-chain assignments
- Measurement of NOE contacts
Backbone Assignments

- Deuteration reduces relaxation
- Maximum sensitivity with 100% deuteration
Backbone Experiments with Deuterated Proteins

• Out and back

• CT for $^{13}$C evolution ($1/J_{cc}$)

• $^1$H T1s:
  - longer recycle delay
  - preserve water

• Deuterium decoupling

• Removal of residual C$\alpha$H

• Sensitivity enhancement (20 nsec limit?)
  Shan et al (1996) JACS 118 6570-79
$2T_c = 26.6 \text{ ms} = 1/J_{cc}$

$\tau_d = 1.7\text{ ms} = 1/4J_{CH}$

- $^2\text{H}$ restored to z-axis (lock stability)
- $C\alpha H$ evolves for $1/2J_{CH}$ - removed by $^1\text{H}$ decoupling
Backbone Assignments

HBCB/HACANNH

HBCB/HACA(CO)NNH

## Isotopomers in 50% $^2$H Proteins

<table>
<thead>
<tr>
<th>Isotopomer</th>
<th>Fraction</th>
<th>Contribution for C$\beta$ peaks</th>
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<tbody>
<tr>
<td>C$\beta$H$_2$-C$\alpha$H</td>
<td>12.5%</td>
<td>4%</td>
</tr>
<tr>
<td>C$\beta$HD-C$\alpha$H</td>
<td>25%</td>
<td>16%</td>
</tr>
<tr>
<td>C$\beta$H$_2$-C$\alpha$D</td>
<td>12.5%</td>
<td>17%</td>
</tr>
<tr>
<td>C$\beta$HD-C$\alpha$D</td>
<td>25%</td>
<td>63%</td>
</tr>
<tr>
<td>C$\beta$D$_2$-C$\alpha$H</td>
<td>12.5%</td>
<td>0%</td>
</tr>
<tr>
<td>C$\beta$D$_2$-C$\alpha$D</td>
<td>12.5%</td>
<td>0%</td>
</tr>
</tbody>
</table>
Effect of Deuteration on HBCB/HACA(CO)NH for a protein with $\tau_c \sim 18$ns (30kDa)
Effect of Deuteration on HBCB/HACANNH for a protein with 18 nsec correlation time
Triple Resonance NMR and Random Fractional Deuteration

- Backbone assignments
- Side-chain assignments
- Measurement of NOE contacts
Side-chain Assignments

HCC(CO)NNH

- Deuteration reduces relaxation
- Maximum sensitivity with 50% deuteration
Effect of Deuteration on HCC(CO)NNH for a Protein with a 12 nsec Correlation Time
Side-chain Assignments

- Deuteration reduces relaxation
- Deuteration removes both protons

HCCH-COSY

HCCH-TOCSY
Effect of Deuteration on HCCH-TOCSY for a Protein with 18 nsec Correlation Time

![Valine spectrum](image1)

0%-$^2$H

50%-$^2$H

![Isleucine spectrum](image2)

0%-$^2$H

50%-$^2$H
Isotope Shifts

Isotope effects:

1Δ $^{13}$C ~ -0.25 ppm
2Δ $^{13}$C ~ -0.1 ppm
3Δ $^{13}$C ~ -0.07 ppm
1Δ $^{15}$N ~ -0.3 ppm
2Δ $^{15}$N ~ -0.05 to -0.1 ppm

e.g $^{13}$Cα in 100% D protein -0.5 ppm isotope shift

weak secondary structure dependency.

Isotopomers not resolved in most experiments
Triple Resonance NMR and Random Fractional Deuteration

- Backbone assignments
- Side-chain assignments
- Measurement of NOE contacts
Measurement of NOE Contacts

- Deuteration affects the measurement of HN - HN, HN - HC and HC - HC cross peaks in different ways
Distance Restraints and Fractional Deuteration

$\tau_c = 12 \text{ nsec}$

NH/sidechain

NH/NH

0% $^2$H

50% $^2$H

75% $^2$H
Measurement of NOE Contacts

- Similar contributions from different isotopomers lead to comparable chemical shifts
Structure Calculations

• Use of ARIA for structure determination

• Global fold from selectively protonated data

• Use Talos to estimate backbone torsion angles from chemical shifts
Fractional Deuteration at 50%  

- Improved sensitivity and linewidth
- No complications from different isotopomers
- Backbone and sidechain assignments possible
- $^1\text{H}/^1\text{H}$ NOEs can be observed
- Applicable to proteins up to 30 kDa
Approaches to the Structure Determination of larger Proteins

• For proteins of up to $\tau_c \sim 12$ ns, use $^{13}$C/$^{15}$N-labelling
• For proteins of up to $\tau_c \sim 18$ ns, use fractional deuteration and $^{13}$C/$^{15}$N-labelling
• For proteins $\tau_c \sim 18$ ns and above, use selective protonation and $^{13}$C/$^{15}$N-labelling
Approaches for Proteins Larger than 30 kDa

Complete deuteration
  maximum sensitivity for backbone experiments but limited NOE information (HN <-> HN)

Selective protonation of residues e.g. AILV in deuterated background

Selective protonation of Methyl-groups
  (Gardner J. Am. Chem. Soc. 119, 7599)
Triple Resonance NMR and Selective Protonation

- Label proteins with ILV+FY
- Methyl and aromatic $^{13}$C and $^1$H nuclei relax more slowly
  - Allows measurement of NH-NH, NH-methyl and methyl-methyl NOE contacts
- These residues are typically found in the protein core or interfaces
- Biosynthetic pathways allow straightforward labelling
- Can adjust the number of residue types labelled
Triple Resonance NMR and Selective Protonation

- Backbone assignments
- Side-chain assignments
- Measurement of NOE contacts
Backbone Assignments

- Deuteration reduces relaxation
- Maximum sensitivity with 100% deuteration
- ILV - Cα mainly deuterated
- Me-selective - Cα 100% deuterated

HNCA

HN(CO)CA
Triple Resonance NMR and Selective Protonation

- Backbone assignments
- Side-chain assignments
- Measurement of NOE contacts
Side-chain Assignments

- Deuteration reduces relaxation
- Maximum sensitivity with 100% deuteration
Side-chain Assignments

\((H)CC(CO)NNH\)

Triple Resonance NMR and Selective Protonation

• Backbone assignments
• Side-chain assignments
• Measurement of NOE contacts
Measurement of NOE Contacts

- 3D $^{15}\text{N}$- and $^{13}\text{C}$-NOESY

- 3D Val/Ile (HM)CMCB(CMHB) NOESY

- 3D $^{13}\text{C}/^{13}\text{C}$-NOESY

- 3D HQQF-NOESY
Methyl-Selective Correlation Experiments

DEPT-HQQC

(Kessler, 1989)

HQQF

all $^1H$ transverse (16 msec)

$\xi_2$

$\phi_2$

$\phi_3$

$\phi_1$

$\phi_{rec}$

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Sensitivity Improvement in HQQF

(a) HQQF
(b) HQQC
(c) DEPT-HQQC
(c) QQF-HSQC

$^1H$ (ppm)
Resolution Improvement in HQQF Cdc42 methyl region
Structure Calculations

• Use ARIA for structure determination

• Restrain $\phi$ and $\varphi$ angles in early stages and slacken off later
Limited Restraints in Structure Determination

Ras:
• Mixed $\alpha$ and $\beta$ structure
• Reasonable size (21kDa)
• Single domain

Numbers of Methyls and Aromatics:

11 Ile
11 Leu
15 Val
5 Phe
9 Tyr
0 Trp
NHs only - 13Å RMSD

HN/NH$_2$
Ile, Val, Leu
Aromatic
Structures from Limited Restraints

Ras NMR Structure
0.6Å RMSD
101 HN-HN
1171 HN-S/C
1862 SC/SC

$^{13}$C/$^{15}$N ILVFY
2.0Å RMSD
101 HN-HN (ass-ass)
390 HN-S/C (ass-amb)
431 S/C-S/C (ass-ass)

$^{13}$C/$^{15}$N ILV $^{15}$N FY
5.0Å RMSD
101 HN-HN (ass-ass)
390 HN-S/C (ass-amb)
231 ILV-ILV (ass-ass)
174 ILV-FY (ass-amb)
26 FY-FY (amb-amb)

$^{13}$C/$^{15}$N ILV $^{15}$N FY
1.7Å
NH NOEs to 5Å
As above with 330 HN-HN NOEs
Structures from Limited Restraints: Effect of Dipolar Couplings

Clore et al (1999) JACS 121 6513-14

BAF - 89 residues all $\alpha$
CVN - 101 residues 2 domains - all $\beta$

<table>
<thead>
<tr>
<th></th>
<th>BAF</th>
<th>CVN</th>
</tr>
</thead>
<tbody>
<tr>
<td>$HN-HN$</td>
<td>106</td>
<td>101</td>
</tr>
<tr>
<td>$HN-Me$</td>
<td>40</td>
<td>84</td>
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<tr>
<td>$HN$-arom</td>
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<td>18</td>
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<td>$Me-Me$</td>
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<td>70</td>
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<tr>
<td>$Me$-arom</td>
<td>51</td>
<td>53</td>
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<tr>
<td>arom-arom</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>245</td>
<td>331</td>
</tr>
<tr>
<td>$Hbonds$</td>
<td>37</td>
<td>40</td>
</tr>
<tr>
<td>$RDC$</td>
<td>259</td>
<td>334</td>
</tr>
</tbody>
</table>

H-bonds + $HN$ + Me ~4Å accuracy
Adding aromatics - 1.37Å (BAF) 1.53Å (CVN)
Adding RDC - 0.91Å (BAF) 1.10Å (CVN)

RDC: $HN,N-C',HN-C',C\alpha-H,C\alpha-C'$
Conclusions

• Selective Protonation
  – optimises the sensitivity of experiments used to correlate side-chain and backbone resonances
  – allows one to obtain limited NOE data and structural models
  – use of orientational restraints should improve structures
Practical Aspects of Producing Deuterated Proteins

- Random fractional deuteration

LB/H₂O → LB/50% D₂O

M9 in 50% D₂O with either
[50% ²H, 100% ¹³C/¹⁵N] algal hydrolysate or [100% ²H/¹³C] glucose

OD₆₀₀ = 0.4
Practical Aspects of Producing Deuterated Proteins

- Selective protonation
- Use $^2\text{H}/^{13}\text{C}$ glucose, amino acids and:

\[
\begin{align*}
\text{CH}_3\text{-CD}_2\text{-CO-COO-} \ [3,3-^2\text{H}]^{13}\text{C}-2\text{-ketobutyrate} \\
\text{CH}_3\text{-CD}_2\text{-CD(CD}_3\text{)-CD(ND}_3^+\text{-COO-} \text{ isoleucine}
\end{align*}
\]


\[
\begin{align*}
\text{LB/H}_2\text{O} \rightarrow \text{LB/95-99}\% \text{ D}_2\text{O} \\
\text{OD}_{600} = 0.4
\end{align*}
\]

M9 in 95-99\% D$_2$O

- [100\% $^2$H/$^{13}$C] glucose
- [100\% 3,3-$^2$H$_2$, 100\% $^{13}$C] 2-ketobutyrate
- [100\% $\alpha/\beta-^2$H, 100\% $^{13}$C/$^{15}$N] Val