Group Epitope Mapping by Saturation Transfer Difference NMR To Identify Segments of a Ligand in Direct Contact with a Protein Receptor

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Aim

• Confirmation that GEM can be investigated by STD NMR studies
  
  – GEM for $\beta$-GalOMe
  – GEM for $\text{NA}_2$
  – Determining the effect of ligand excess on STD NMR
  – Determination of competitive binding
Group Epitope Mapping (GEM)

• Relevant for the understanding of binding systems:
  – Cellular recognition
  – Drug-receptor complexes
  – Signal transduction processes
  – Colloidal matrices
Fig. 1 The mechanism of STD experiments:

a) On-resonance, b) Off-resonance, grey) Difference spectrum

GEM is based on the proximity of ligand protons to the receptor core
GEM – Past and Present

- Historically achieved via X-Ray crystallography

- Before the widespread use of STD NMR
  - trNOE
  - SAR by NMR

- These NMR techniques complement STD NMR
STD Advantages

- Direct identification of the binding component
- Identification from a mixture of compounds \( (K_D = 10^{-3} - 10^{-8} \, M) \)
- Suitable for HTS
- Epitope mapping based on NMR signal Intensity.
- Highly sensitive, 1nmol limit for proteins >10KDa
- No isotopic labeling required (native states)
- Ligand choice relatively wide
  - Carbohydrates, peptides, glyopeptides, drug candidates
- Can be coupled with other spectroscopic techniques
  - TOCSY, HQSC, NOESY
Binding system studied

• Protein Receptor:
  – *Ricinus Communis* Agglutinin I, RCA$_{120}$

• Ligands:
  – Methyl β-D-galactopyranoside, β-GalOMe
  – Biantennary decasaccharide, NA$_2$
**RCA$_{120}$**

- *Ricinus Communis* Agglutinin I (RCA$_{120}$)
  - Castor Bean

- Lectin family
  - Tetramer
  - 2 As-sB dimers
    - B-chain: lectin domain

- Affinity for terminal β-D-galactosyl residues
  - binding prevents attachment to carbohydrates in the cell membrane.

Fig. 2 A lectin, derived from Jack Bean
The ligands studied

- **NA$_2$**
  - 1836 Da
  - Isolated from fibrinogen

- **β-GalOMe**
  - 194 Da
  - Purchased, not isolated
Experimental conditions

• Measured on:
  – Bruker Avance DRX 500 MHz Spectrometer

• Sample preparation:
  – 500µL D₂O buffer
  – 20mM NaCl, 10mM Phosphate buffer pH 7, 0.04% NaN₃

• Protein conc.
  – Between 20 and 50 µM
  – (UV Abs 280nm, Beer-Lambert Law)

• Ligand added to Protein from stock solutions
STD pulse scheme

- Saturation period:
  - selective pulse, 50 ms
    - (x 40)
    - Gaus
    - Strength 86Hz
  - delay between pulses, 1ms
  - saturation train, 2.04s
  - subtraction (on and off-res) performed via phase cycling after every scan, δ
  - On-res. irradiation, -0.4 ppm
  - Off-res. irradiation, 30 ppm

Fig. 3 Pulse sequence for a typical 1D STD NMR spectra recorded in D$_2$O
STD pulse scheme

- Eliminating background Protein Resonances:
  - $T_{1\rho}$ filter
    - 30-ms-spin-lock pulse
    - (after $\pi/2$ pulse)
    - Strength $(\gamma/2\pi)B_1 = 4960$ Hz

Facilitates clearer analysis

- Reduces ligand STD signal intensity
- Solution: Ref. NMR spectra were also recorded with the same spin-lock pulse

Fig. 4 Pulse sequence for the 1D STD NMR spectra recorded in D$_2$O with an additional $T_{1\rho}$ filter.
STD pulse scheme

- Suppression of residual HDO signal:
- WATERGATE
- Binomial 3-9-19 pulse sandwich
- 2 ms delay between pulses
- Strength $(\gamma/2\pi)B_1 = 6944$ Hz
- Inverts all signals except the HDO signal at the carrier frequency

Fig. 5 Pulse sequence for the 1D STD NMR spectra recorded for H$_2$O samples with an additional $T_{1p}$ filter and WATERGATE.
Steps involved in an STD NMR experiment

A. Ref 1D NMR of 120kDa RCA\textsubscript{120} (50µM in binding sites)

B. Corresponding STD NMR spectrum

C. 1D NMR spectrum with a T\textsubscript{1ρ} filter

D. Ref 1D NMR of RCA\textsubscript{120} and 1.2mM \(\beta\)-GalOMe without T\textsubscript{1ρ} filter

E. Corresponding STD NMR

F. STD NMR spectrum as in E with T\textsubscript{1ρ} filter

Fig. 6 Shows the steps involved in the STD NMR experiment.
Experiments:

• Analysis of β-GalOMe
  – GEM
  – Titration
  – Ligand excess

• Analysis of NA₂.
  – GEM
  – 1D STD Experiment
  – 2D STD TOCSY Experiment
  – Ligand excess

• Comparative analysis of NA₂ and β-GalOMe
  – Ligand excess
  – Competition Studies
GEM; Analysis of β-GalOMe

Fig. 7 Left: (A) Ref. WATERGATE NMR spectrum of a mixture of RCA120 (40 µM binding sites) and β-GalOMe (4 mM) in a ratio of 1:100. (B) WATERGATE STD NMR spectrum of the same sample. Above Right: Relative saturation of Protons in β-GalOMe

- STD spectrum proves binding
- Ligand protons nearest to the Protein identified
- Binding epitope characterised using relative integral STD signal intensities.
- H3 reference, set to 100%
- H2 and H4 87%
- H5, H6a, H6b approx. 63-67%
- H1 and OMe H, 40 and 32%
Fig. 8 (A) Diagram showing the fraction of the H4 signal of β-GalOMe which is saturated at a given ligand excess. The concentration of RCA120 was 40 μM and the saturation time 2 s. (B) Display of the same data in terms of the STD amplification factor. This second plot shows that even though the fraction of ligand which is saturated decreases at a higher ligand excess, the absolute STD signal intensity increases in the form of a saturation curve.

**STD effect:** \[ \eta_{STD} = \frac{I_o - I_{sat}}{I_o} = \frac{I_{STD}}{I_o} \]

**STD amplification factor:** \[ A_{STD} = \frac{I_o - I_{sat}}{I_o} \times \text{ligand excess} \]
Ligand excess; Analysis of β-GalOMe

Fig. 9 Observed STD amplification factors of two resonances of β-GalOMe plotted against the saturation time $T_{\text{sat}}$ at three different ligand concentrations (9, H3 proton; b, OMe protons). STD amplification factors at concentrations of (A) 0.5 mM, (B) 1 mM, and (C) 4 mM β-GalOMe in the presence of 40 μM binding sites of RCA120. A large ligand excess yields larger STD intensities and better discrimination between strongly and weakly binding groups.

Relative saturation of Protons in β-GalOMe
GEM, Analysis of NA$_2$.

1D STD Experiment

- most intensive STD signals
  - Gal-6/6’
  - GlcNAc-5/5’

- The spectral region, 3.65 to 3.75 ppm strong STD signals
  - From H5 and H6a/6b of Gal 6/6’ and the H2, H3, and H4 of GlcNAc-5/5’ protons in equal parts

- almost no detectable STD signal int.
  - H1-Fuc-1’ and R-H1-GlcNAc-1

Fig. 10 (A) Section of a reference NMR spectrum of a mixture of RCA120 (50µM binding sites) and NA2 (0.55 mM) in a ratio of 1:11. (B) STD NMR spectrum revealing that the directly interacting residues of NA2 have the strongest signals.
GEM, Analysis of NA₂.  
2D STD TOCSY Experiment

- STD spectrum B:
  - strong traces corresponding to:  
    - H1-Gal-6/6’ and H1-GlcNAc-5/5’
  - reduced intensities:  
    - H1-Man-4 and H1-Man-4’
    - due to further distance to the binding site of RCA120.
  - cross-peaks absent:  
    - GlcNAc-1/2 and Fuc-1’
    - proving that they have no interaction with the protein.

Fig. 11 (A) Reference or off-resonance TOCSY spectrum of NA2.  
(B) STD TOCSY spectrum obtained by subtraction of an on-resonance TOCSY spectrum from spectrum A.
Ligand excess; Analysis of \( \text{NA}_2 \).

Fig. 12 Titration plot of \( \text{NA}_2 \) to NMR sample containing RCA120 (20 M in binding sites), monitoring the increase of the STD amplification factor of the H4-Gal-6/6’ proton versus the ligand concentration \( (T_{\text{sat}}) \) 2 s).
Ligand excess;
Comparative analysis of NA$_2$ and β-GalOMe

Table 1. STD Signal Intensity of NA2 and β-GalOMe at a Ligand excess of 12.5- and 100-Fold, Respectively

<table>
<thead>
<tr>
<th>NA$_2$ (β-GalOMe) resonance</th>
<th>11-fold excess NA$_2$ (%)</th>
<th>12.5-fold excess β-GalOMe (%)</th>
<th>100-fold excess β-GalOMe (%)</th>
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</thead>
<tbody>
<tr>
<td>H4-Gal-6/6$^b$</td>
<td>~100</td>
<td>90</td>
<td>87</td>
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<tr>
<td>(H4-β-GalOMe)</td>
<td></td>
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<tr>
<td>H3-Gal-6/6$^b$</td>
<td>~100</td>
<td>100</td>
<td>100</td>
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<td>(H3-β-GalOMe)</td>
<td></td>
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<td></td>
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<tr>
<td>H2-Gal-6/6$^b$</td>
<td>~100</td>
<td>95</td>
<td>87</td>
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<td>(H2-β-GalOMe)</td>
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<td></td>
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<tr>
<td>H1-Gal-6/6$^b$</td>
<td>70</td>
<td>61</td>
<td>40</td>
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<tr>
<td>(H1-β-GalOMe)</td>
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<tr>
<td>H5-Gal-6/6$^b$</td>
<td>~80</td>
<td>74</td>
<td>67</td>
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<td>(H5-β-GalOMe)</td>
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</tr>
<tr>
<td>H6a/6b-Gal-6/6$^b$</td>
<td>~80</td>
<td>72</td>
<td>63</td>
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<td>(H6a/6b-β-GalOMe)</td>
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<tr>
<td>H2, H3, H4-GlcNAc-5/5$^b$</td>
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<td>(H1-GlcNAc-5/5$^b$</td>
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<td>H2-Man-4</td>
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<td>H2-Man-4$'$</td>
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<td>H2-Man-3</td>
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<td>NHAc-5/5</td>
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<td>NHAc-2</td>
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<td>H6-Fuc-1$'$</td>
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</table>

- Ligand excess on STD effects:
- Differentiation between direct and indirect contact could have been more pronounced at a higher excess for NA$_2$
- H1- β-GalOMe
  - 61% at 12.5 fold excess
  - 40% at 100% fold excess
Competition Studies

Fig. 12 Left: Diagram showing the STD amplification factors H1 β-GalOMe; H1 Gal-6/6′ NA2) determined from STD spectra on titration of β-GalOMe to a sample of RCA120 (50 µM in binding sites) and NA2 (0.55 mM). The STD amplification factor of the signal corresponding to NA2 decreases from 1 to 0.66 with increasing concentration of β-GalOMe. This competition experiment gives evidence for the specificity of the RCA120 toward galactose-containing saccharides. The $K_D$ of NA2 can be calculated to be 27 µM.

Right: The STD amplification factors of selected cross-peak intensities of NA2 and β-GalOMe determined from STD TOCSY spectra. The selected cross-peaks represent the F1 traces of the two H1 protons of the galactose residues. These values are consistent with those obtained from the 1D STD spectra therefore, even a few cross-peaks can be sufficient to perform titration experiments.
Conclusions

• STD NMR spectroscopy:
  – analyzing binding processes
  – screening libraries
  – mapping of ligand epitopes

• The use of a high ligand excess is advantageous:
  – signal intensities are larger, making the STD experiment more sensitive
  – Differentiation between direct and indirect contact could have been more pronounced at a higher excess

• Determination binding epitope
  – integrals of the signals in 1D spectra
  – 2D cross-peak integrals
References


Improvements and future outlooks

• Regarding cell-peptide interactions:
  – Second generation saturation transfer double difference (STDD)
    • Cell and membrane protein interactions can be studied

• Host-guest interactions:
  – Group selective STD ($^{15}$N GS STD NMR)
    • Reduces signal overlap

• Receptor-small molecule interactions:
  – Clean STD NMR
    • 3 x more sensitive