Assessment of the Higher Order Structure of Monoclonal Antibody Therapeutics by NMR: Present and Future Directions

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Health Canada Santé Canada

Canada

People in the NMR laboratory

Geneviève Gingras

From Genes to Magnet: Molecular biology, production of labeled proteins (*E. coli* and *Pichia Pastoris*)

Derek Hodgson

Protein expression, NMR data collection

Dr. Houman Ghasriani

From Samples to Structures: NMR data collection and analysis, structure determination and dynamics studies

Assessment of the Higher Order Structure of Protein Therapeutics : Biosimilars

For the approval of biosimilars, guidance documents have been developed by most regulatory agencies (European Union, Canada, Japan, United States and others).

The common guiding principle: only a reduced set of clinical trials will be required, provided that similarity is demonstrated with a thorough comparability exercise with a recognized comparator.

The comparability exercise includes an array of physico-chemical tests and biological assays to assess the similarity of various quality attributes between product and comparator.

Assessment of the Higher Order Structure of Protein Therapeutics

Amongst the set of critical quality attributes (CQA), the higher order structure (HOS) is the most important one

Actual methods used to assess the secondary and tertiary structure of recombinant protein therapeutics:

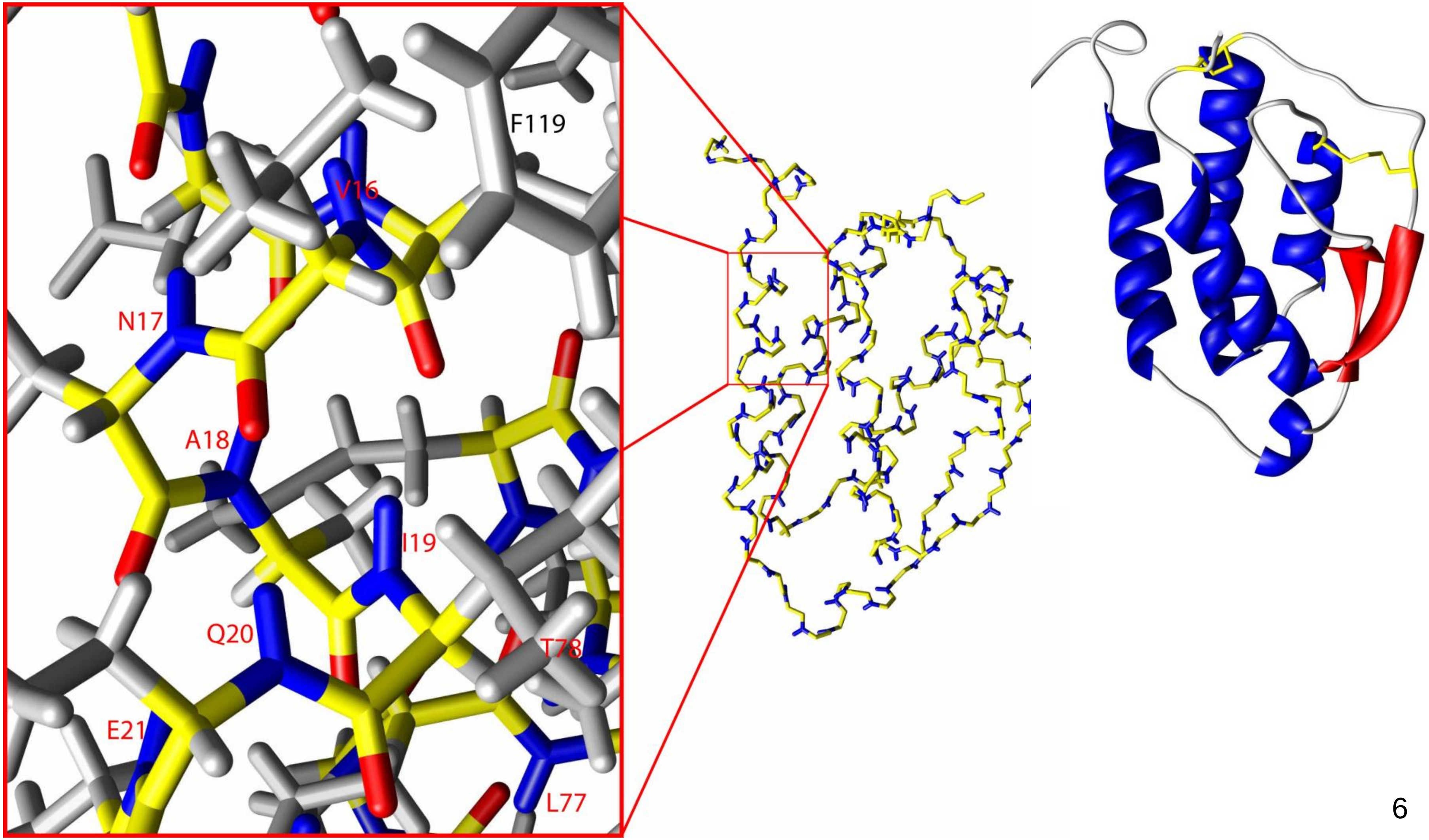
Circular Dichroism (2^o)
Fourier Transform Infra Red Spectroscopy (3^o)

These yield low resolution information.

Assessment of the Higher Order Structure of Protein Therapeutics

Proposed methodology is based on simple NMR spectroscopy techniques to assess the bioactive conformation of a recombinant protein therapeutic.

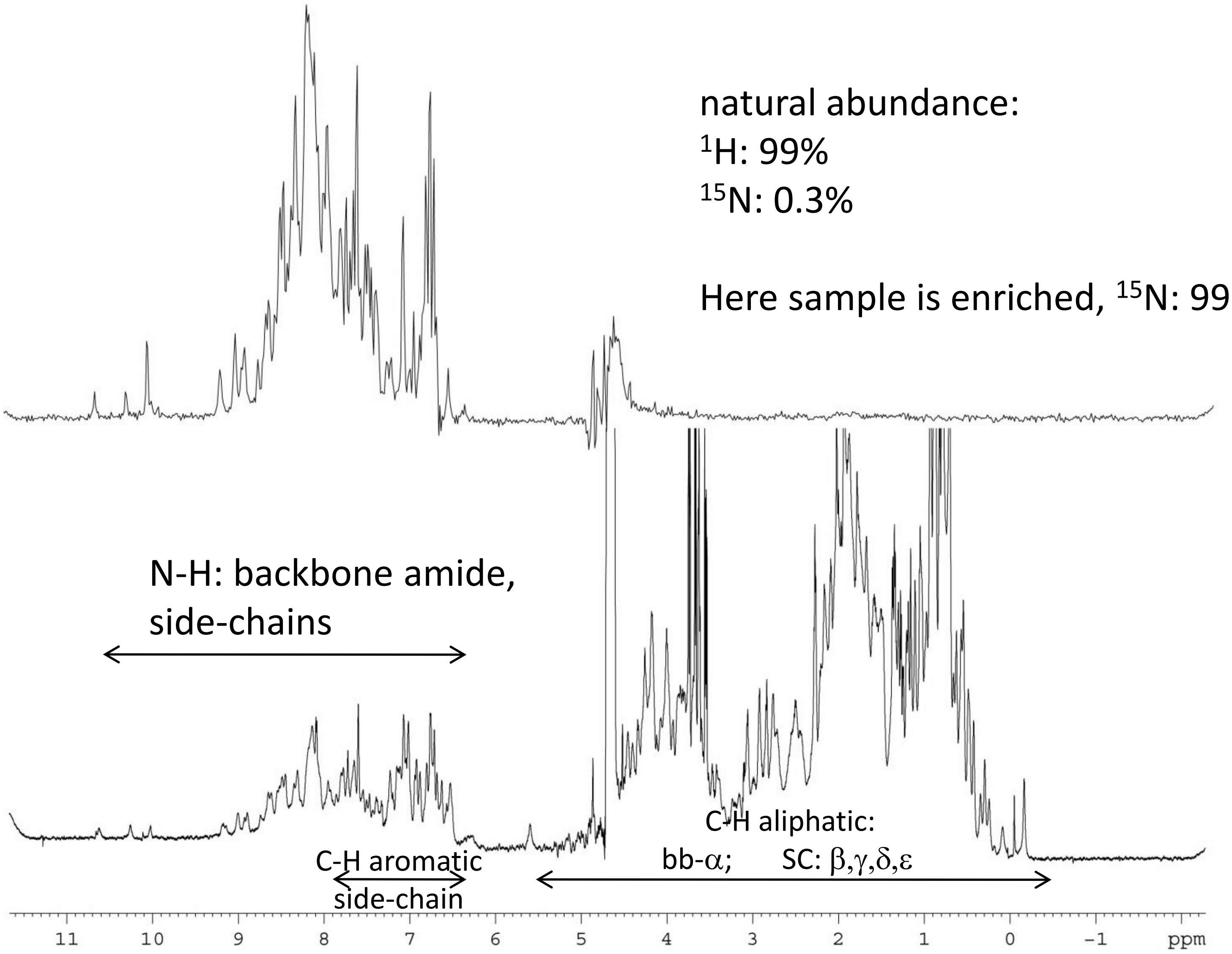
Change in Local Magnetic Environment =
Change in Local Conformation



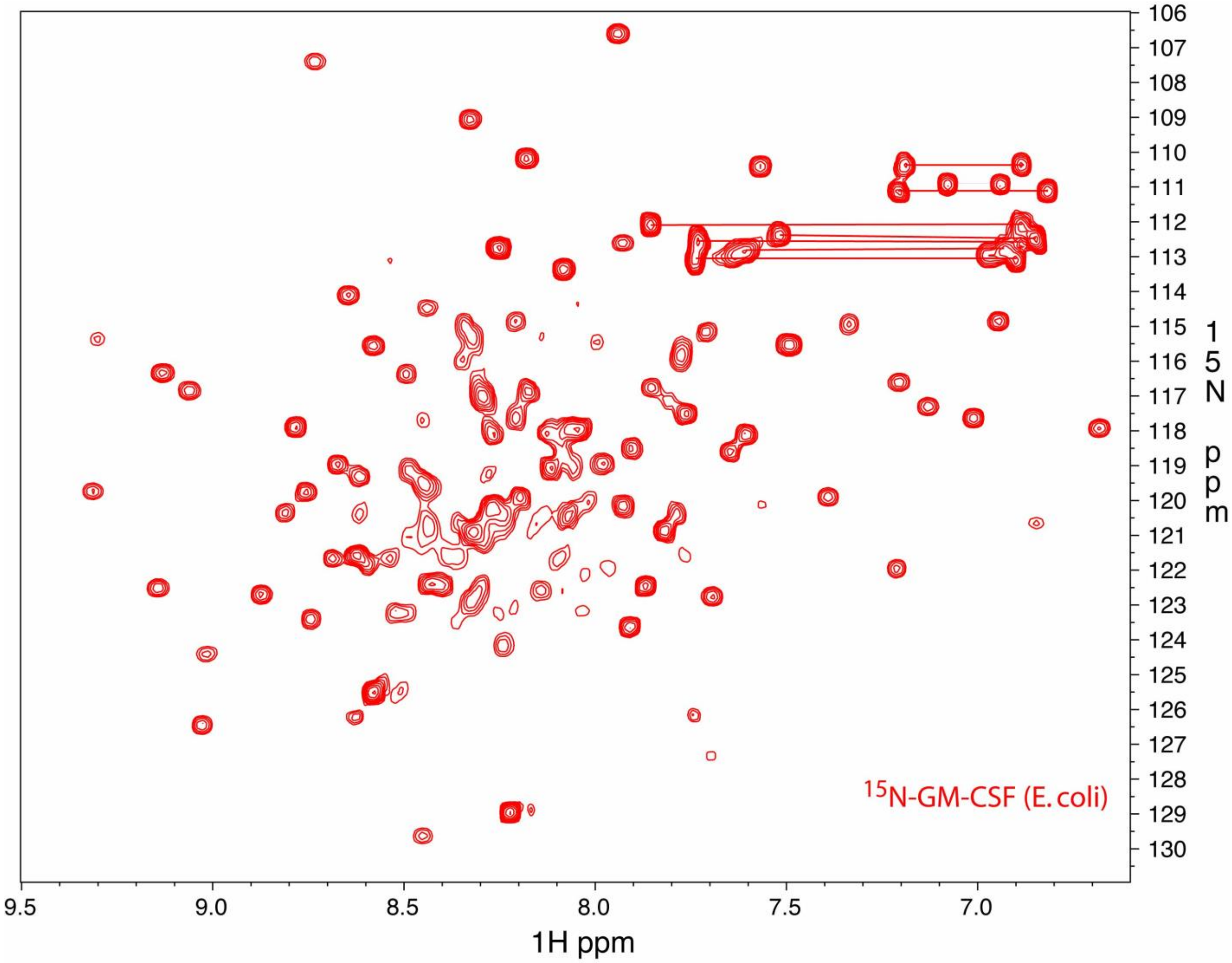
1D-^{1}H- and ^{1}H,^{15}N-HSQC of rhGM-CSF

natural abundance:
^{1}H: 99%
^{15}N: 0.3%

Here sample is enriched, ^{15}N: 99%



2D ^{1}H,^{15}N-HSQC rhGM-CSF



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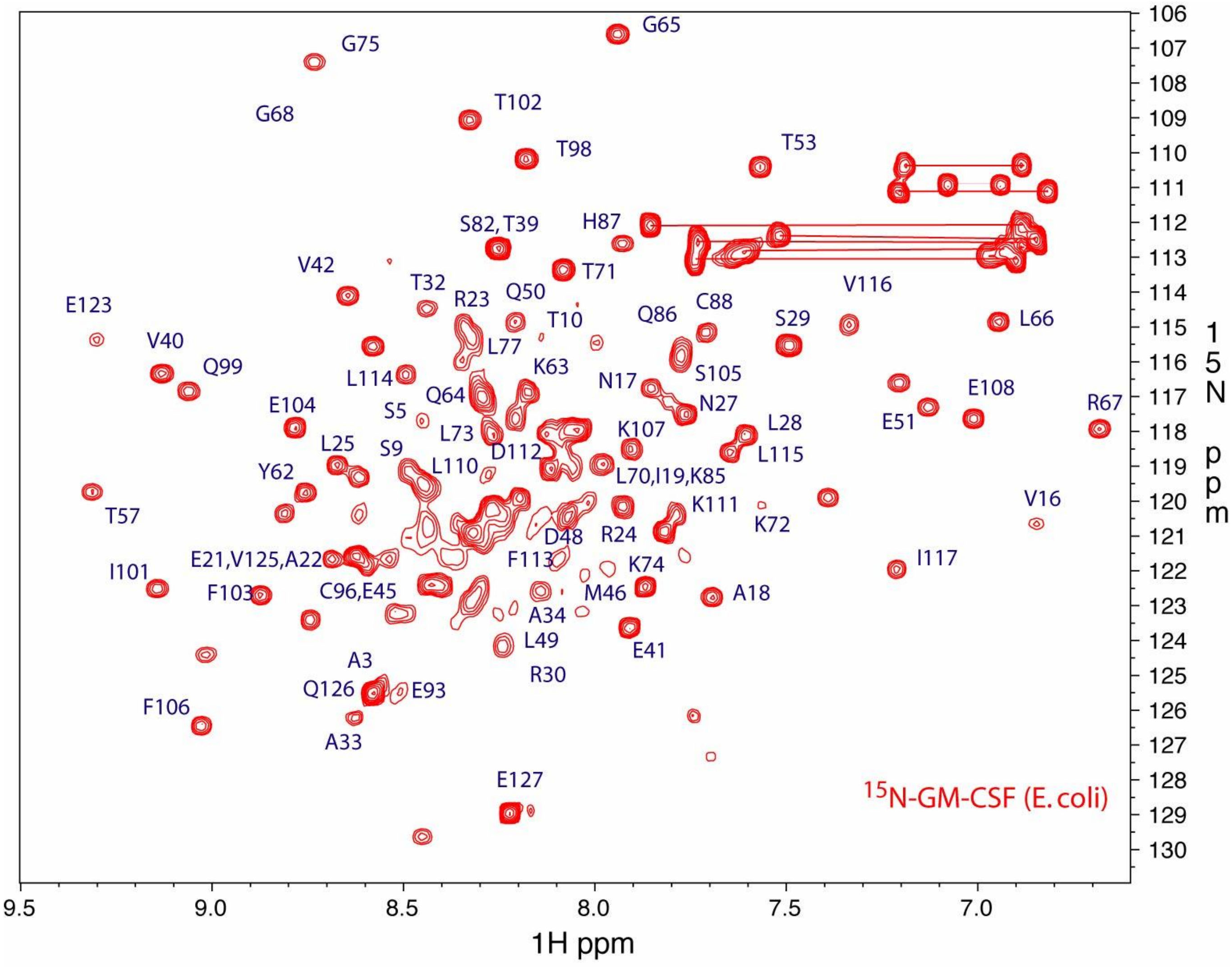
2D $^{1}H,^{15}N$-HSQC rhGM-CSF



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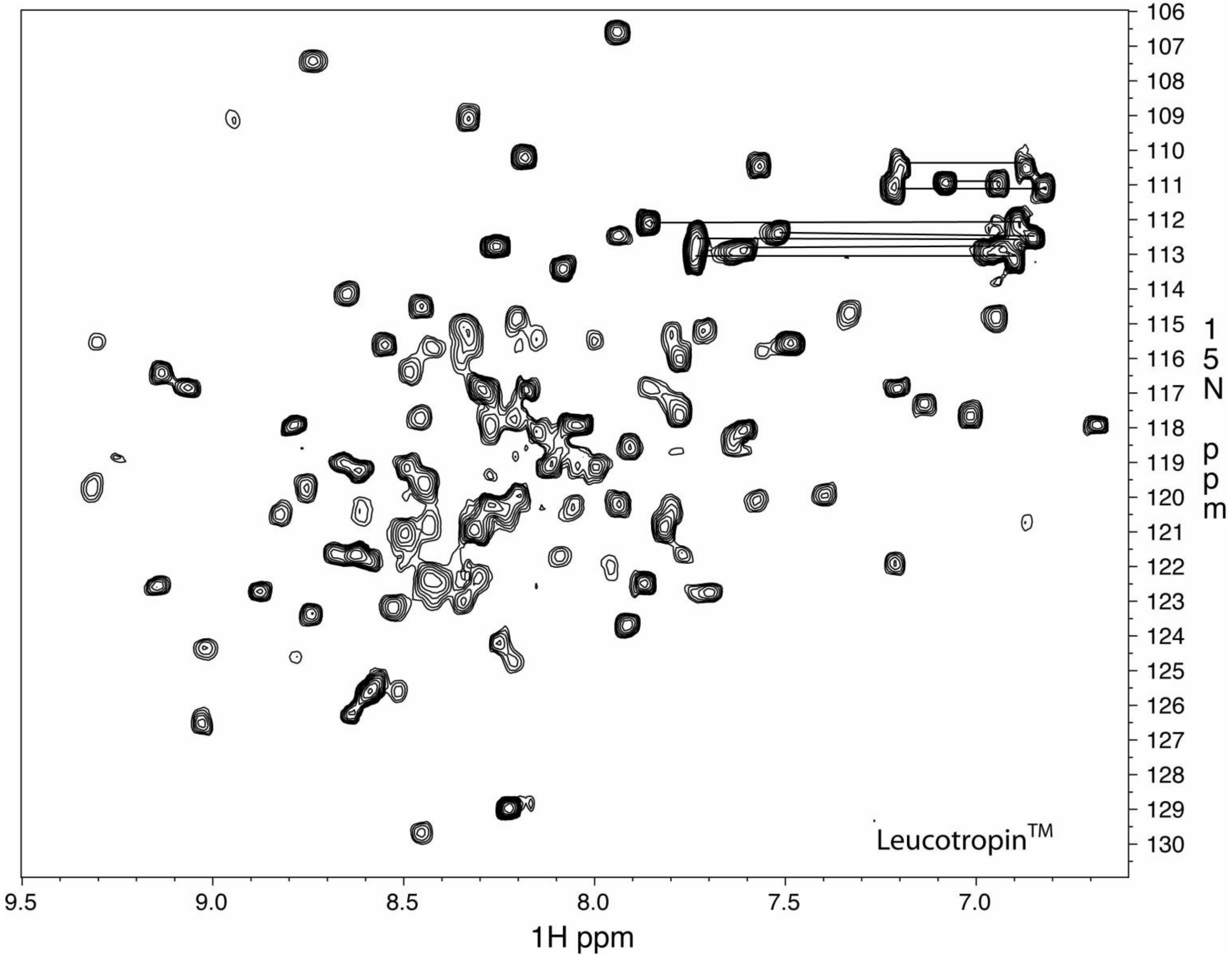
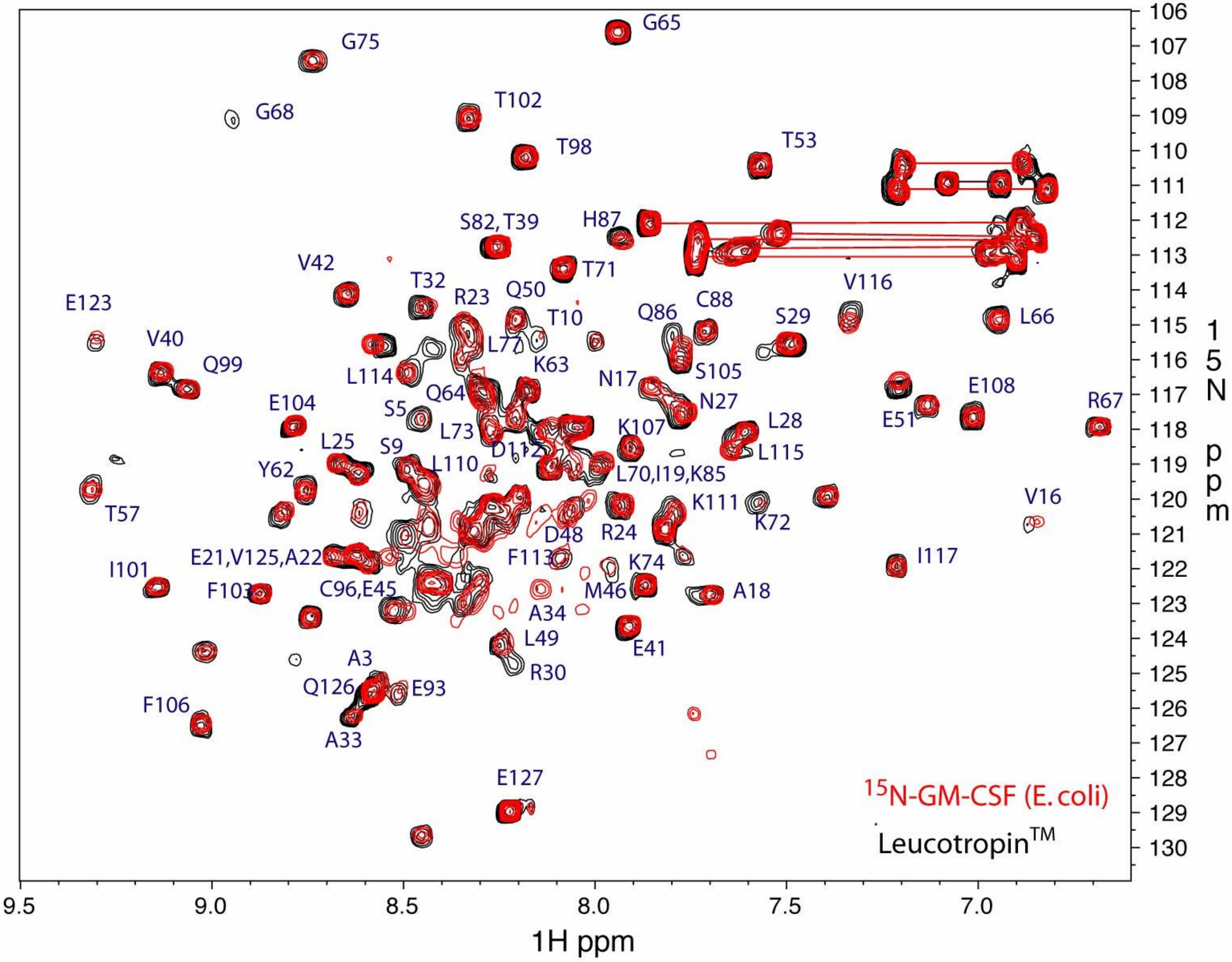
2D ^{1}H,^{15}N-HSQC Leucotropin^{TM}

Natural Abundance

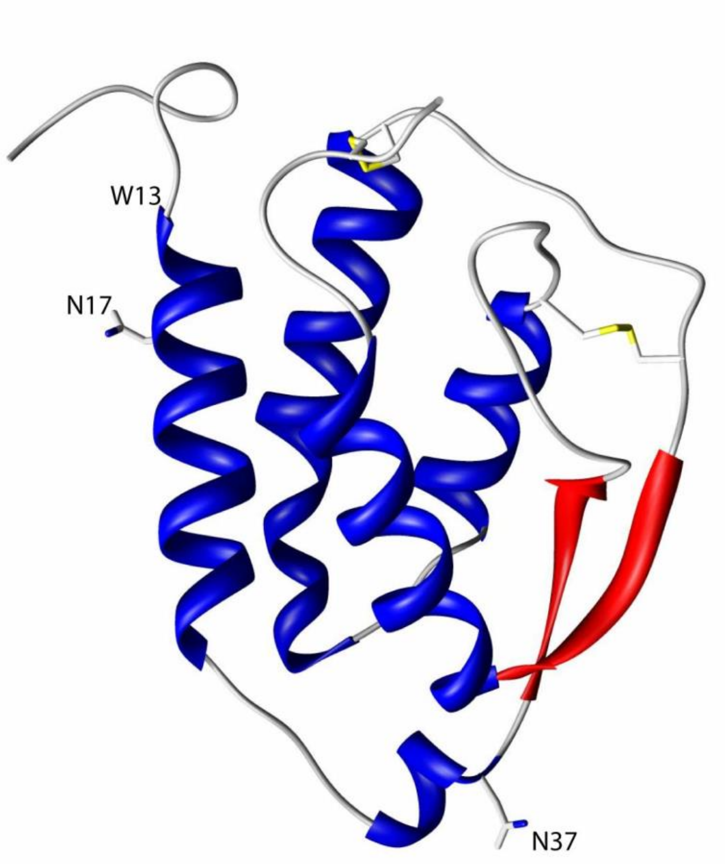


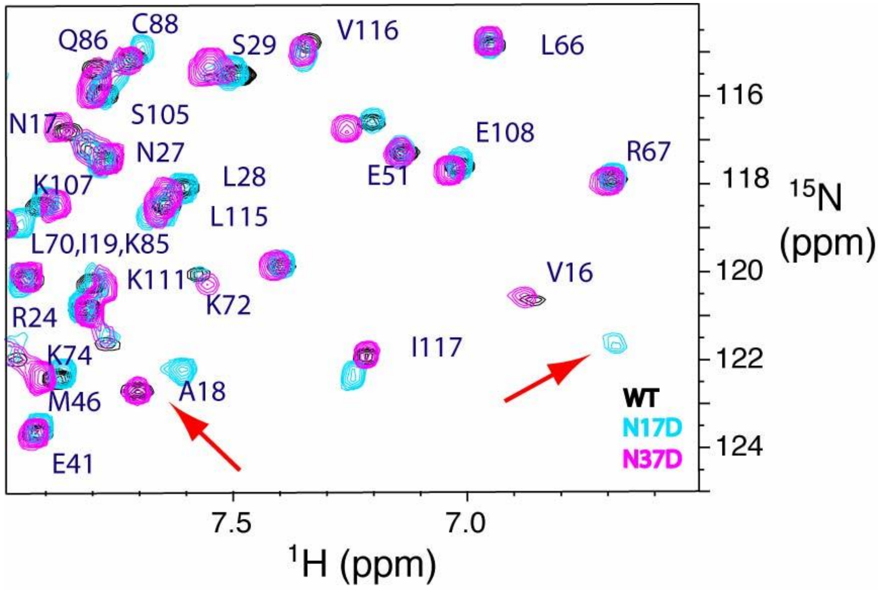
2D ^{1}H,^{15}N-HSQC rhGM-CSF and Leucotropin™

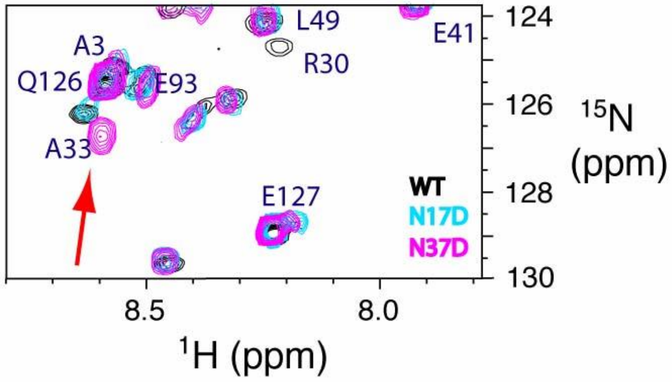
(Aubin et al Anal. Chem 2008)



N17D and N37D induce specific chemical shift pertubations







Summary: When a protein folds, it folds!

Using a single 2D-NMR spectrum, the NMR fingerprinting method allowed the assessment of the **bioactive conformation** of a recombinant protein therapeutic at **<u>amino acid resolution</u>**:

> Leucotropin^{TM} (secreted) and rhGM-CSF (refolded) share an identical conformation

The current sensitivity of the method allows the analysis of samples at 250 μM protein concentration

Reported in *Anal. Chem.* (2008), **80**, 2623-2627

Approved Therapeutic mAbs in US or EU

(as of November 2014)

1. Abthrax (raxibacumab)	IgG1-lambda
2. Actemra (tocilizumab)	IgG1-kappa
3. Adcetrisc (brentuximab vedotin)	IgG1- ADC
4. AlprolIXd (Factor IX Fc fusion protein)	IgG1-Fc + Factor IX
5. Arcalystf (rilonacept)	IgG1-Fc + IL1R
6. Arzerra (ofatumumab)	IgG1-kappa
7. Avastin (bevacizumab)	IgG1-kappa
8. Benlysta (belimumab)	IgG1-lambda
9. Cimziag (certolizumab pegol)	Fab’-PEG2MAL 40K
10. Cyramza (ramucirumab)	IgG1-kappa
11. Eloctateh (Factor VIII Fc fusion protein)	IgG1-Fc + Factor VIII
12. Enbrel (etanercept)	IgG1-Fc + TNFR
13. Entyvio (vedolizumab)	IgG1-kappa
14. Erbitux (cetuximab)	IgG1-kappa
15. Eyleaj (aflibercept)	IgG1-Fc + VEGF
16. Gazyva (obinutuzumab)	IgG1-kappa
17. Herceptin (trastuzumab)	IgG1-kappa
18. Humira (adalimumab)	IgG1-kappa
19. Ilaris (canakinumab)	IgG1-kappa
20. Inflectrak l (infliximab [biosimilar])	IgG1-kappa
21. Kadcylan (ado-trastuzumab emtansine)	IgG1-kappa + emtansine (ADC)
22. Keytruda (pembrolizumab)	IgG4-kappa
23. Lemtrada (alemtuzumab)	IgG1-kappa
24. Lucentiso (ranibizumab)	Fab from IgG1-kappa
25. Nplatep (romiplostim)	IgG1-Fc + peptide (trombopoeitin receptor)
26. Nulojixq (belatacept)	IgG1-Fc + CTLA-4
27. Orenciar (abatacept)	IgG1-Fc + CTLA-4
28. Perjeta (pertuzumab)	IgG1-kappa
29. Prolias (denosumab)	IgG2-kappa
30. Remicade (infliximab)	IgG1-kappa
31. Removabt (catumaxomab)	IgG2ab (rat-mouse hybrid)
32. Remsimak l (infliximab [biosimilar])	IgG1-kappa
33. ReoProu (abciximab)	Fab fragment
34. Rituxan (rituximab)	IgG1-kappa
35. Simponi/ Simponi Aria (golimumab)	IgG1-kappa
36. Simulect (basiliximab)	IgG1-kappa
37. Soliris (eculizumab)	IgG2/4-kappa
38. Stelara (ustekinumab)	IgG1-kappa
39. Sylvant (siltuximab)	IgG1-kappa
40. Synagis (palivizumab)	IgG1-kappa
41. Tysabri (natalizumab)	IgG4-kappa
42. Vectibix (panitumumab)	IgG2-kappa
43. Xgevas (denosumab)	IgG2-kappa
44. Xolair (omalizumab)	IgG1-kappa
45. Yervoy (ipilimumab)	IgG1-kappa
46. Zaltrapw (ziv-aflibercept)	IgG1-Fc + VEGF
47. Zevalinx (ibritumomab tiuxetan)	IgG1-kappa + linker + Yttrium-90

Dawn M Ecker, Susan Dana Jones, and Howard L Levine (2015) *The therapeutic monoclonal antibody market*, mAbs 7:1, 9—14

Pushing the Limit:
NMR of Therapeutic mAbs

Arbogast, Brinson and Marino demonstrated the application of NMR spectroscopy to obtain high-resolution 2D spectra of mAb fragments (Fab and Fc)

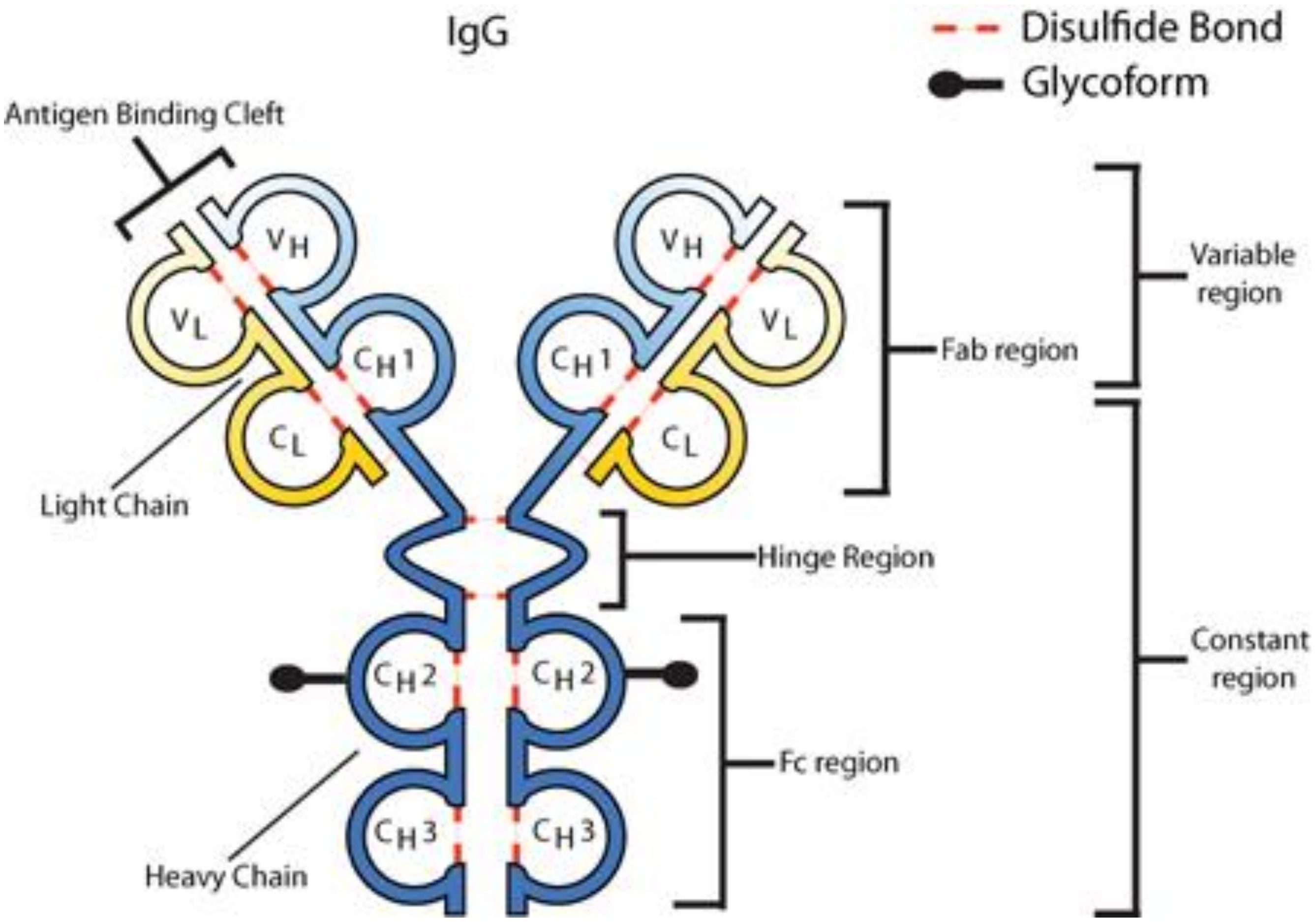
Arbogast, LW, Marino, JP, Brinson, RG (2015) *Analytical Chemistry*, *87*: 3556-3561

Arbogast, LW, Brinson, RG, Formolo, T, Hoopes JT, Marino, JP (2016) *Pharmaceutical Research*, 33:462-475

Therapeutic mAbs

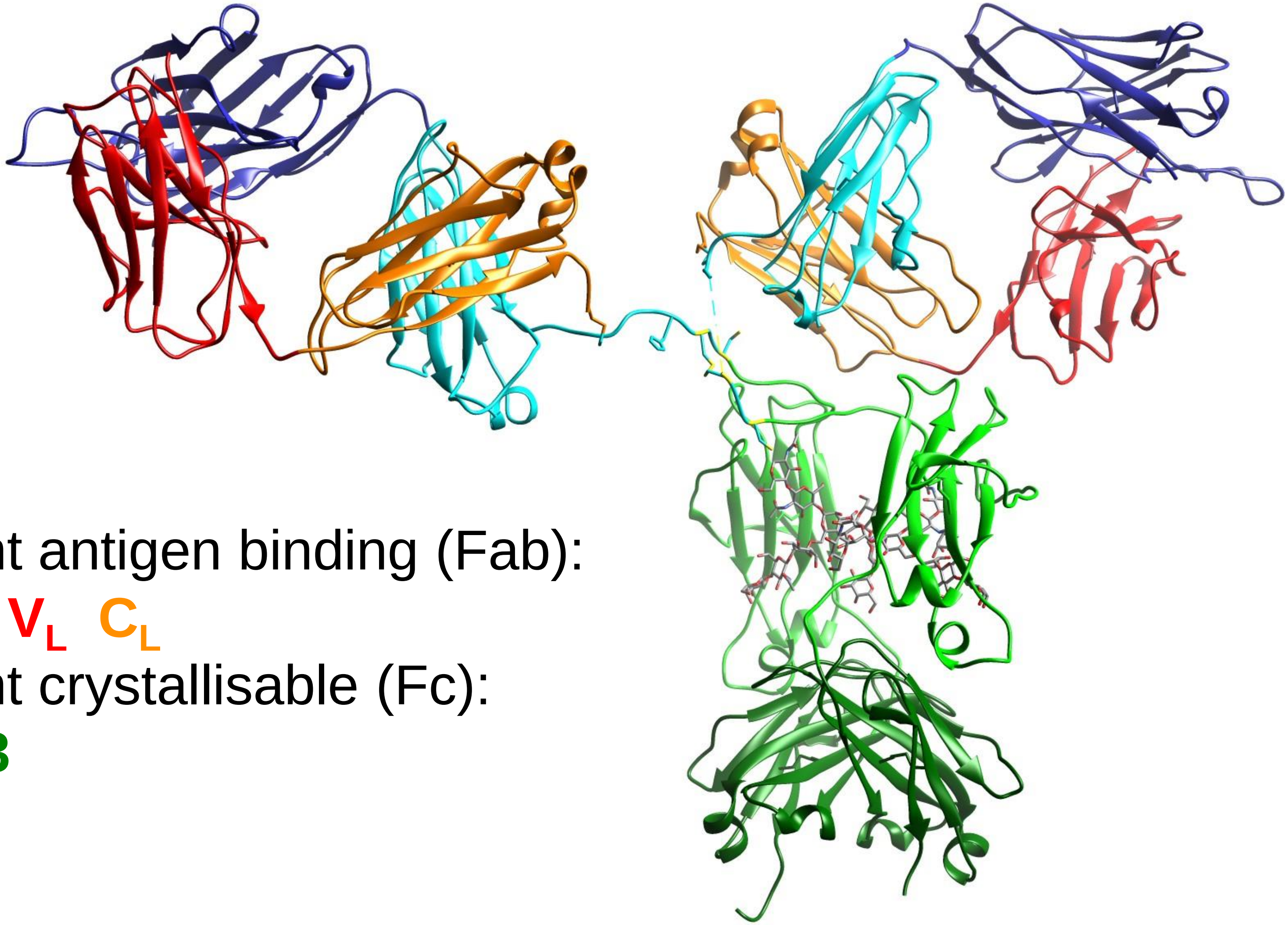
Avastin (bevacizumab)	IgG1-kappa
Rituxan (rituximab)	
Remicade (infliximab)	
Herceptin (trastuzumab)	
Humira (adalimumab)	
Enbrel (etanercept)	IgG1-Fc + TNFR

Therapeutic mAbs



Scott BJ, Klein AV, and Wang J (2014) J. Clin. Pharmacol.

Therapeutic mAbs



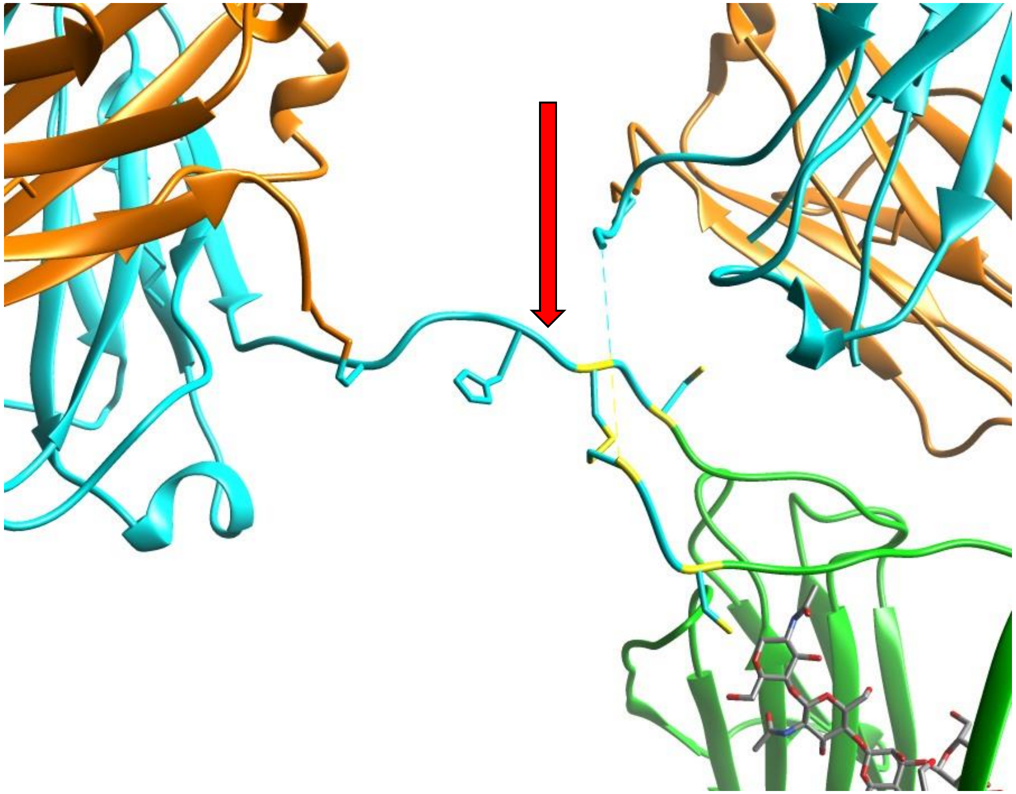
Fragment antigen binding (Fab):

V_H C_H1 V_L C_L

Fragment crystallisable (Fc):

C_H2 C_H3

Papain cleaves after His between Fab and Fc



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Samples Preparation

Papain cleavage using bulk enzyme immobilized on resin

Fab: recovered after Protein A column

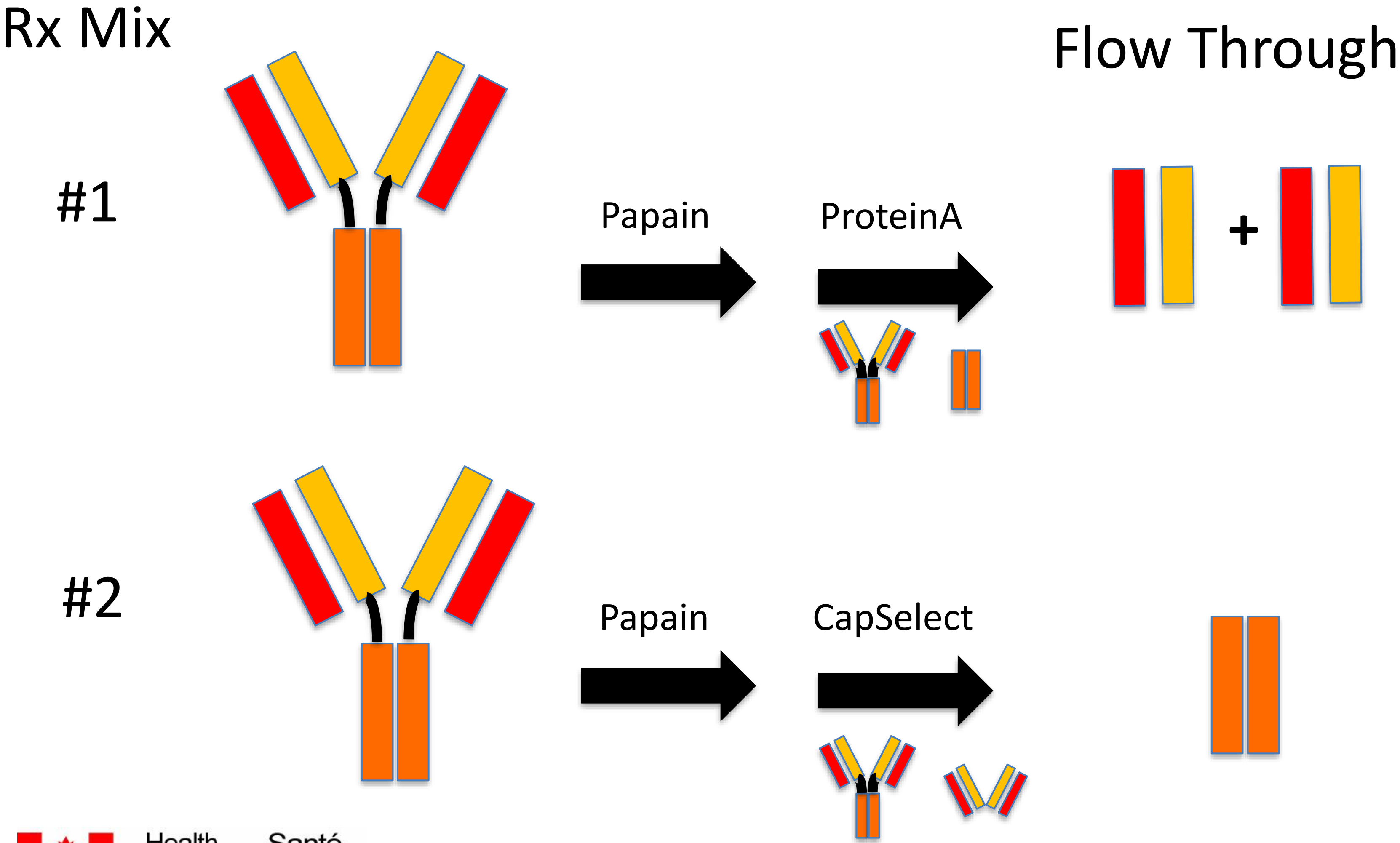
Fc: recovered after Capture Select™ column

Column were re-used ~ 5-7 times

All samples were analyzed in 50 mM Acetate-d3, pH 5.77

Hodgson DJ, Ghasriani H, Aubin Y. J Pharm Biomed Anal. 2018 Oct 1;163:144-152

Sample Preparation



Rx Mix

Flow Through

#1

Papain

ProteinA

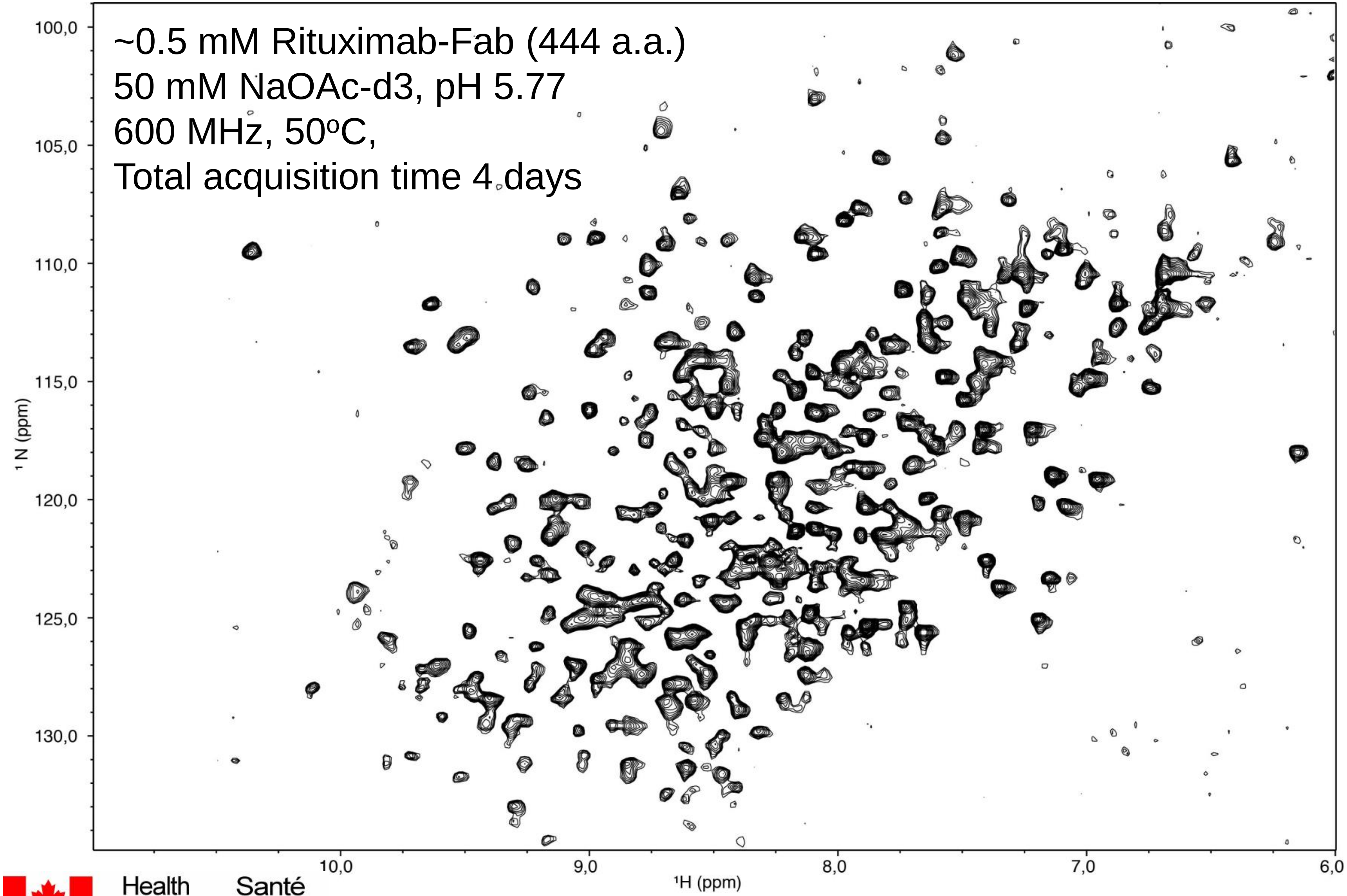
+

#2

Papain

CapSelect

2D-NMR of Rituximab-Fab 600 MHz



~0.5 mM Rituximab-Fab (444 a.a.)
50 mM NaOAc-d3, pH 5.77
600 MHz, 50^{o}C,
Total acquisition time 4 days

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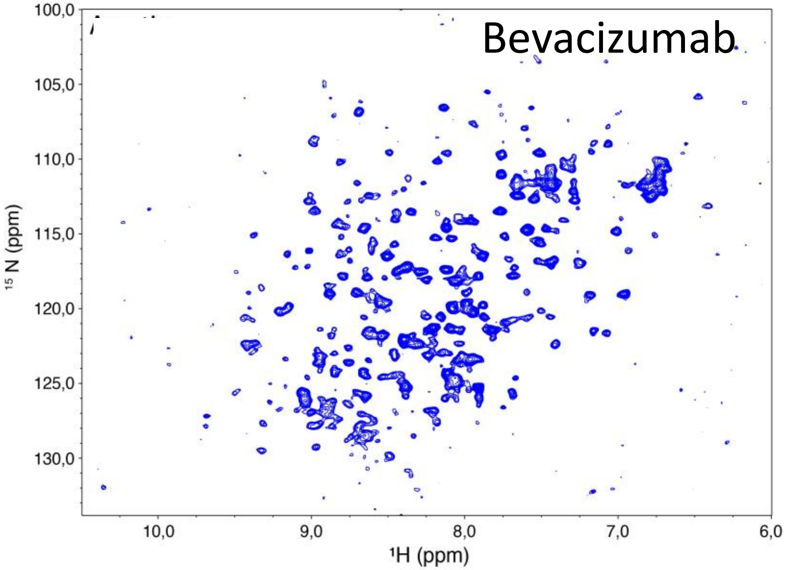
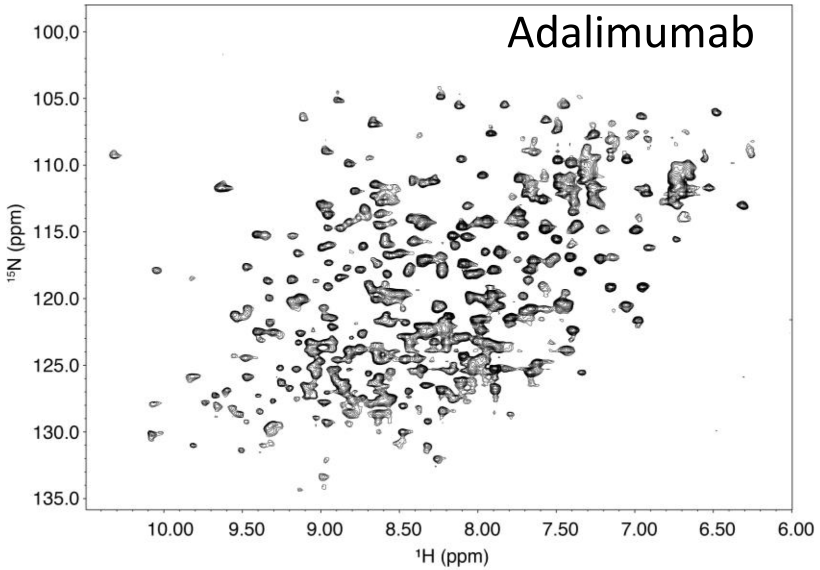
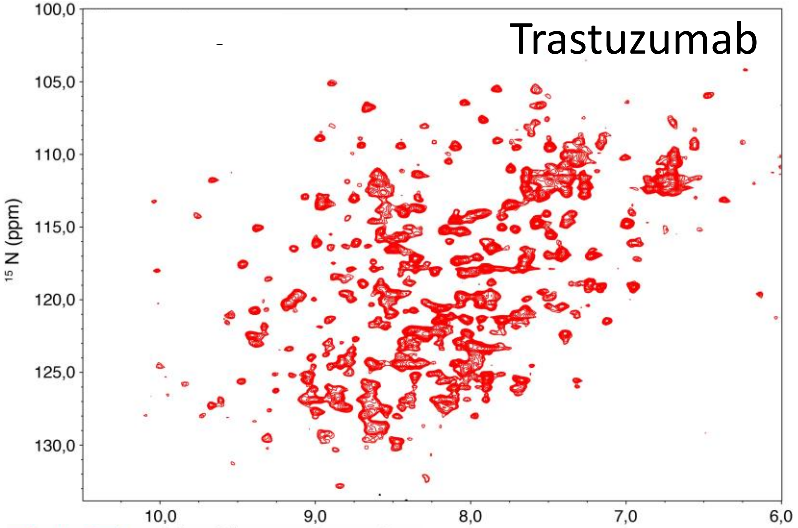
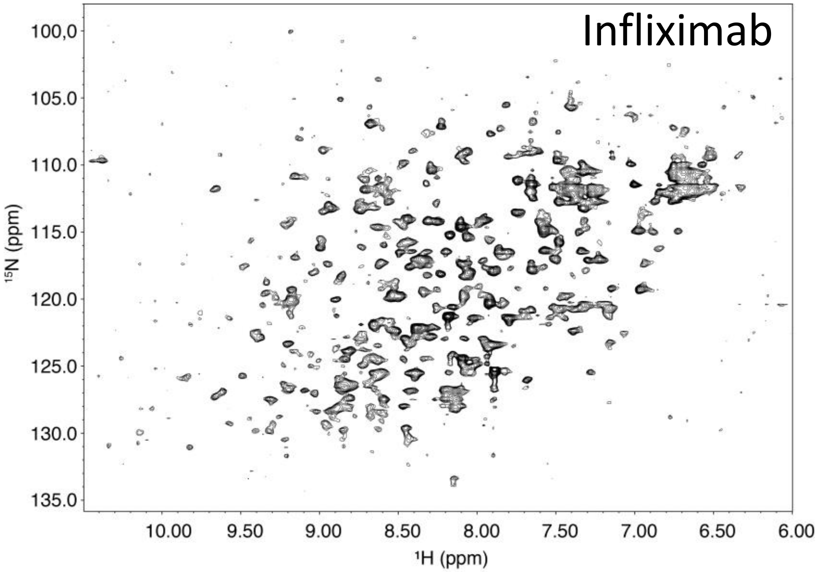
Hodgson DJ, Ghasriani H, Aubin Y. J Pharm Biomed Anal. 2018 Oct 1;163:144-152

2D-^{1}H,^{15}N-NMR of four Fab 700 MHz









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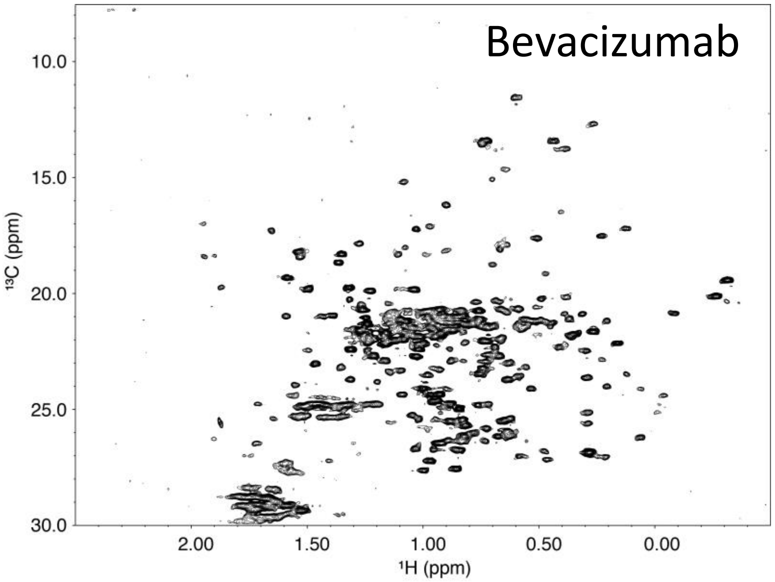
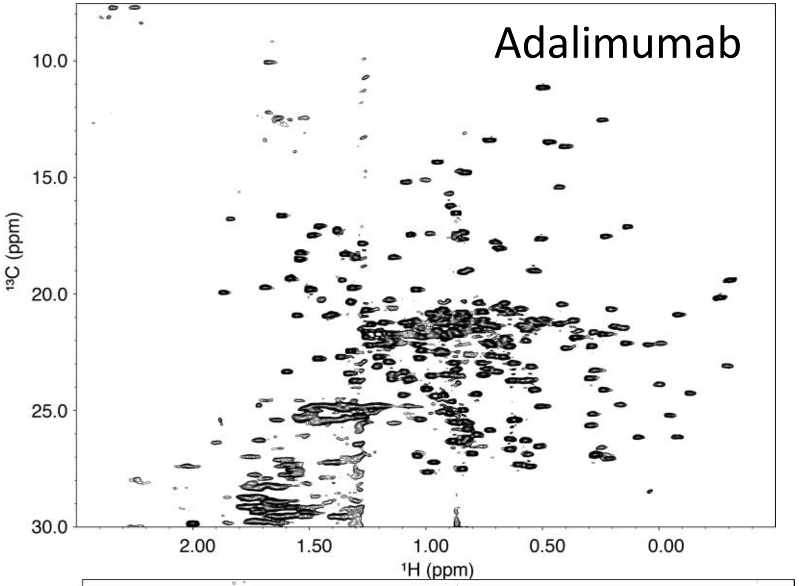
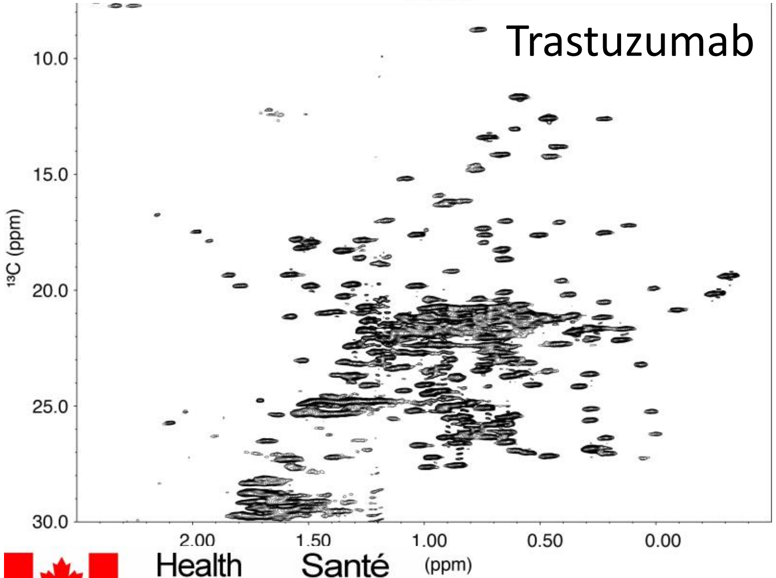
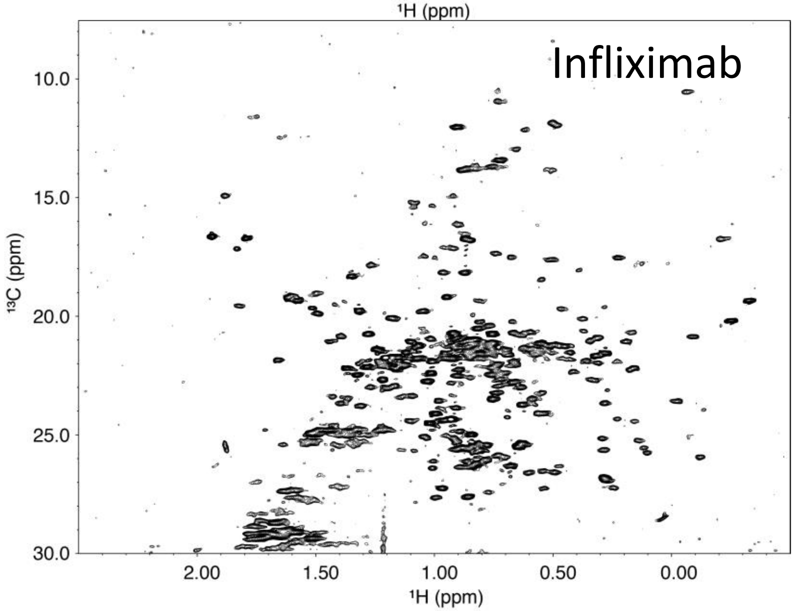
Hodgson DJ, Ghasriani H, Aubin Y. J Pharm Biomed Anal. 2018 Oct 1;163:144-152

2D-$^{1}H^{13}C$-NMR of four Fab 700 MHz









Hodgson DJ, Ghasriani H, Aubin Y. J Pharm Biomed Anal. 2018 Oct 1;163:144-152

Fc sequence alignment

```
Fc domain : (NMR  BMRB 25224) Domain CH2 Domain CH3

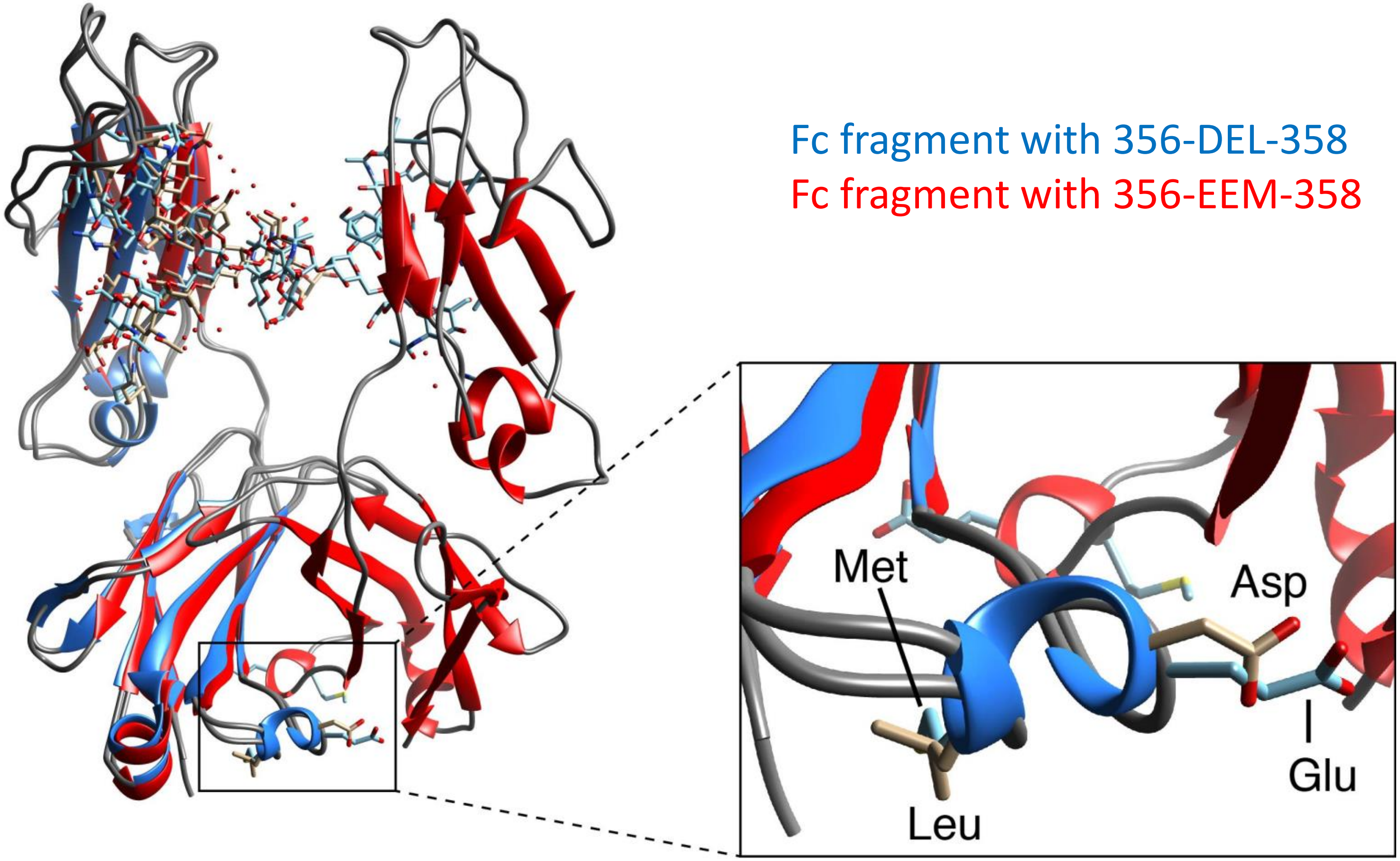
Ritux : TCPPCPAPELLGGPSVFLF PPKPKDTLMISRTPEVTCVV VDVSHEDPEVKFNWYVDGVE VHNAKTKPREEQYNSTYRVV SVLTVLHQDWLNGKEYKCKV
NMR   : TCPPCPAPELLGGPSVFLF PPKPKDTLMISRTPEVTCVV VDVSHEDPEVKFNWYVDGVE VHNAKTKPREEQYNSTYRVV SVLTVLHQDWLNGKEYKCKV
Hercp : TCPPCPAPELLGGPSVFLF PPKPKDTLMISRTPEVTCVV VDVSHEDPEVKFNWYVDGVE VHNAKTKPREEQYNSTYRVV SVLTVLHQDWLNGKEYKCKV
Avast : TCPPCPAPELLGGPSVFLF PPKPKDTLMISRTPEVTCVV VDVSHEDPEVKFNWYVDGVE VHNAKTKPREEQYNSTYRVV SVLTVLHQDWLNGKEYKCKV
NIST  : TCPPCPAPELLGGPSVFLF PPKPKDTLMISRTPEVTCVV VDVSHEDPEVKFNWYVDGVE VHNAKTKPREEQYNSTYRVV SVLTVLHQDWLNGKEYKCKV

Ritux : SNKALPAPIEKTISKAKGQ PREPQVYTLPPSRDELTKNQ VSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDG SFFLYSKLTVDKSRWQQGNV
NMR   : SNKALPAPIEKTISKAKGQ PREPQVYTLPPSRDELTKNQ VSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDG SFFLYSKLTVDKSRWQQGNV
Hercp : SNKALPAPIEKTISKAKGQ PREPQVYTLPPSRDELTKNQ VSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDG SFFLYSKLTVDKSRWQQGNV
Avast : SNKALPAPIEKTISKAKGQ PREPQVYTLPPSREEMTKNQ VSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDG SFFLYSKLTVDKSRWQQGNV
NIST  : SNKALPAPIEKTISKAKGQ PREPQVYTLPPSREEMTKNQ VSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDG SFFLYSKLTVDKSRWQQGNV

Ritux : FSCSVMHEALHNHYTQKSLS LSPGK
NMR   : FSCSVMHEALHNHYTQKSLS LSPG
Hercp : FSCSVMHEALHNHYTQKSLS LSPGK
Avast : FSCSVMHEALHNHYTQKSLS LSPGK
NIST  : FSCSVMHEALHNHYTQKSLS LSPGK

DEL-   356-357-358
```

X-ray structure of Fc domains

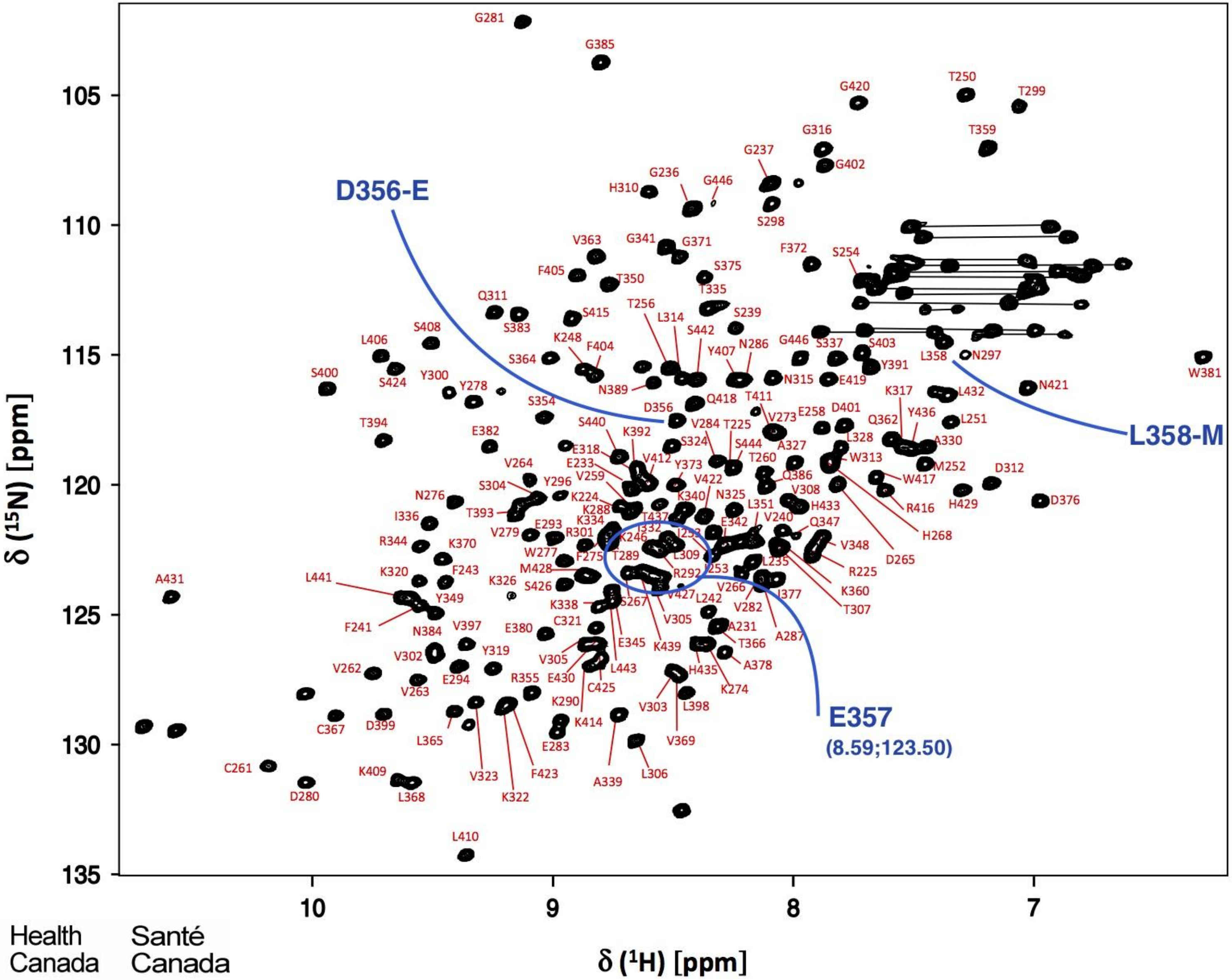


Fc fragment with 356-DEL-358

Fc fragment with 356-EEM-358

Backbone Assignment of Fc

Yagi *et al.* Biomol NMR Assign (2015) 9:257–260



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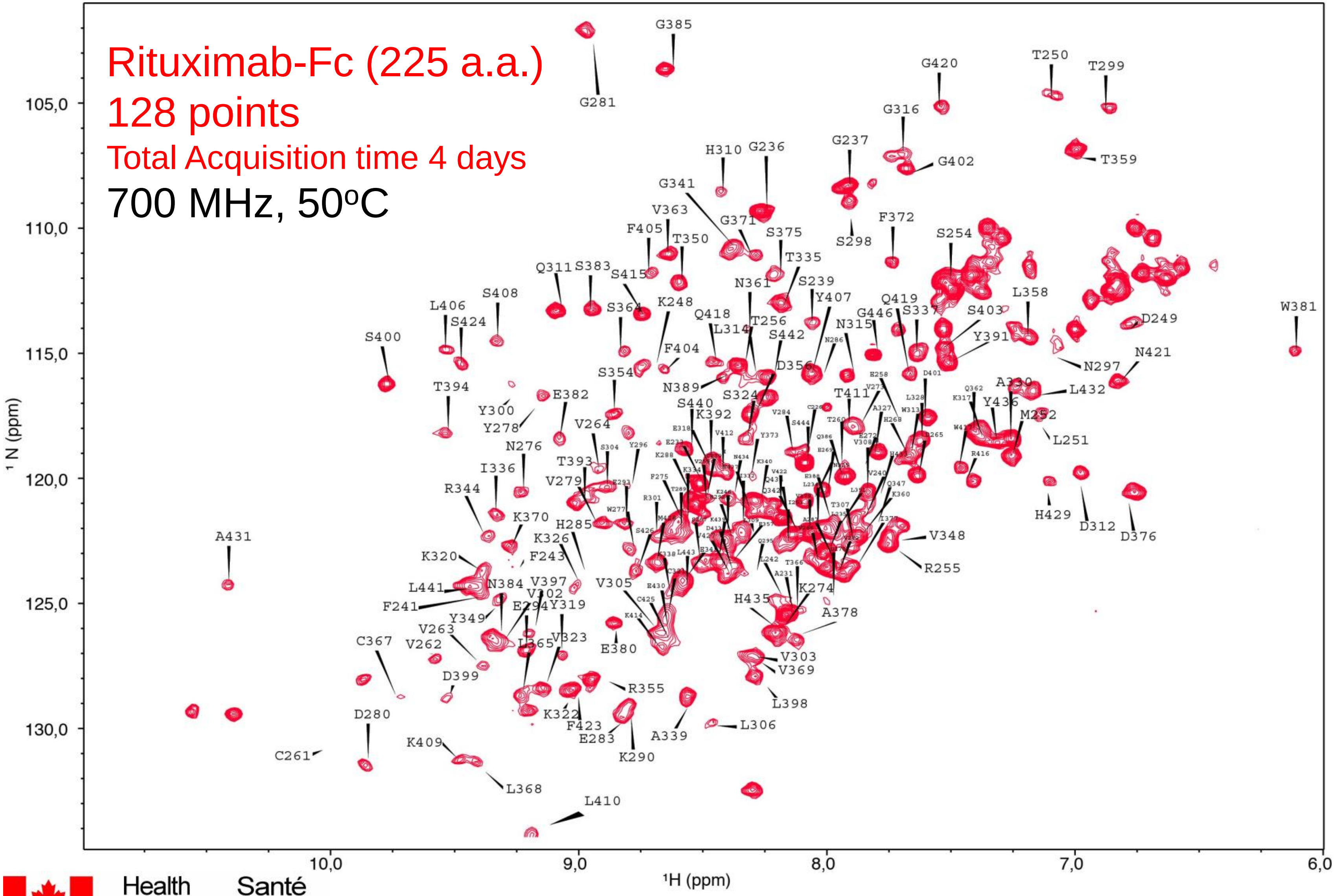
Backbone Assignment of Fc

Yagi *et al.* Biomol NMR Assign (2015) 9:257–260



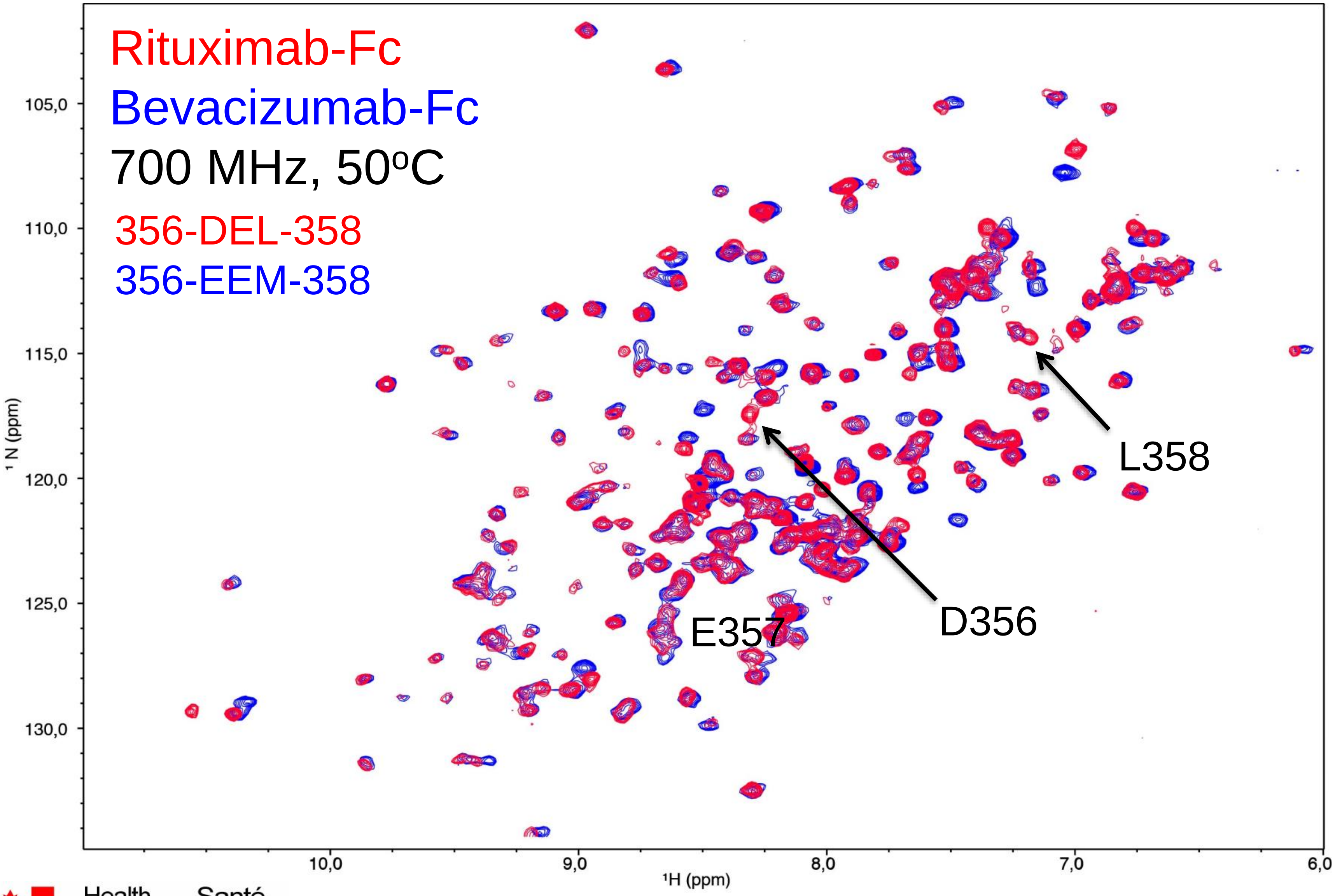
Rituximab-Fc (225 a.a.)

128 points

Total Acquisition time 4 days

700 MHz, 50^{o}C

Two amino acids variants induce several chemical shift difference in the ^{1}H-^{15}N spectrum



Rituximab-Fc

Bevacizumab-Fc

700 MHz, 50°C

356-DEL-358

356-EEM-358

Conclusion

Proton-nitrogen spectra of Fc, it is possible to obtain a significanly higher level of resolution and identify small variations in the primary sequence.

The proton-carbon spectra provide a complementary set of information with higher sensitivity but less resolution.

Future work will aim at improving the sensitivity of the proton-nitrogen spectra.

A Deeper Understanding via Assignment of NMR Resonances

A complete or near complete assignment can be:

A powerful tool to understand the significance (or lack of) of spectral changes with regard to the conformation or the dynamics of the drug substance.

A mean to monitor various perturbations (pH, solution conditions, excipients) at the amino acid level.

Isotopic labeling of NISTmAb Fragments in *Pichia Pastoris*

Fab

Construction of a bis-cistronic vector inserted in the methylotropic *Pichia Pastoris*
Polypeptide is secreted in the culture media after removal of the signal peptide.

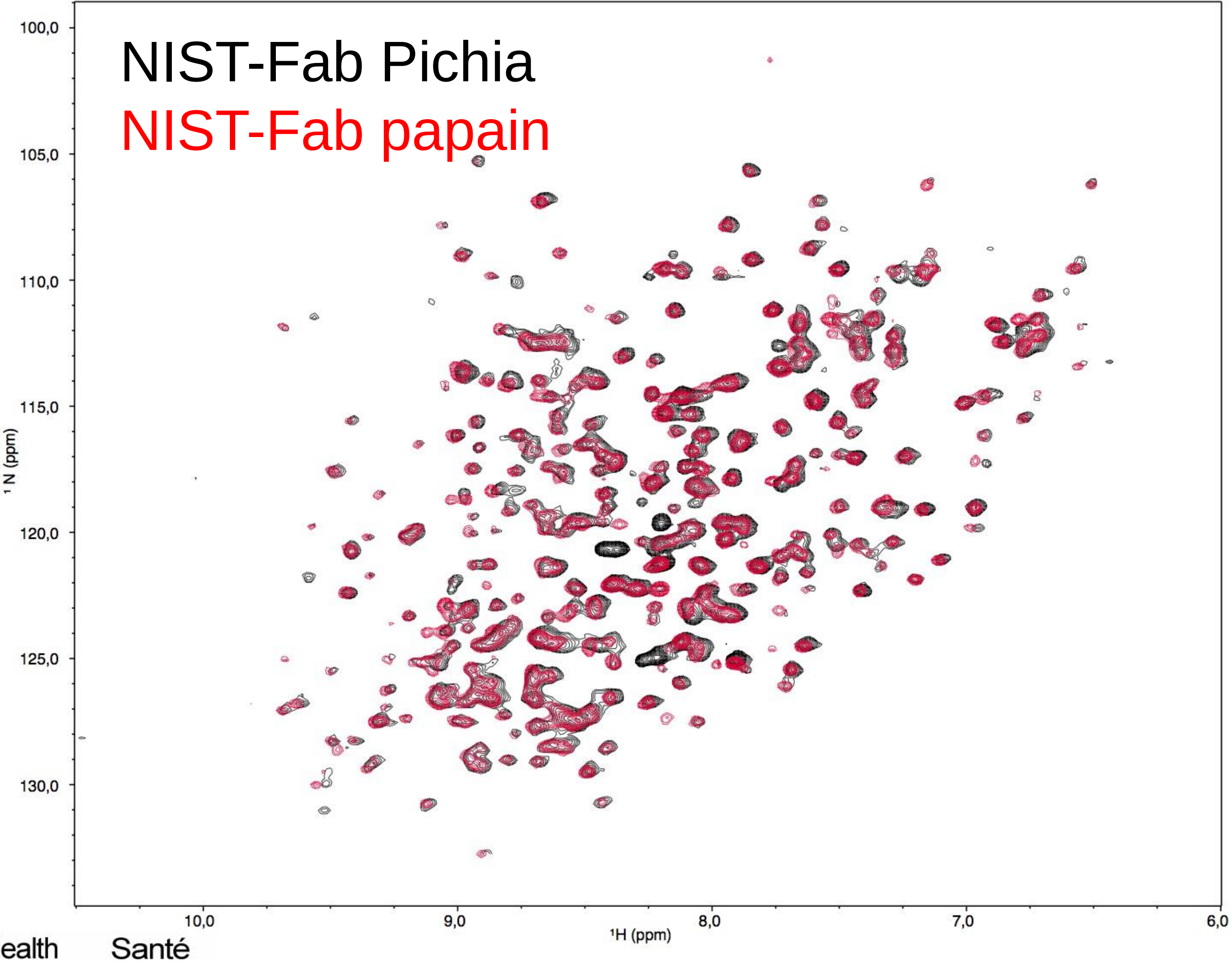
Signal Peptide-EKREAEA –N-ter(Heavy [V_H-C_H1])

Signal Peptide-EKREAEA –N-ter(Light [V_L-C_L])

Fc

Signal Peptide-EKREAEA –N-ter(Heavy [C_H2-C_H3])
The final polypeptide is glycosylated with a high-mannose glycan that is further hyper mannosylated (Glycan MW is ~5000 Da by SDS-PAGE).

Isotopic labeling of NISTmAb Fragments in *Pichia Pastoris*


NIST-Fab Pichia

NIST-Fab papain

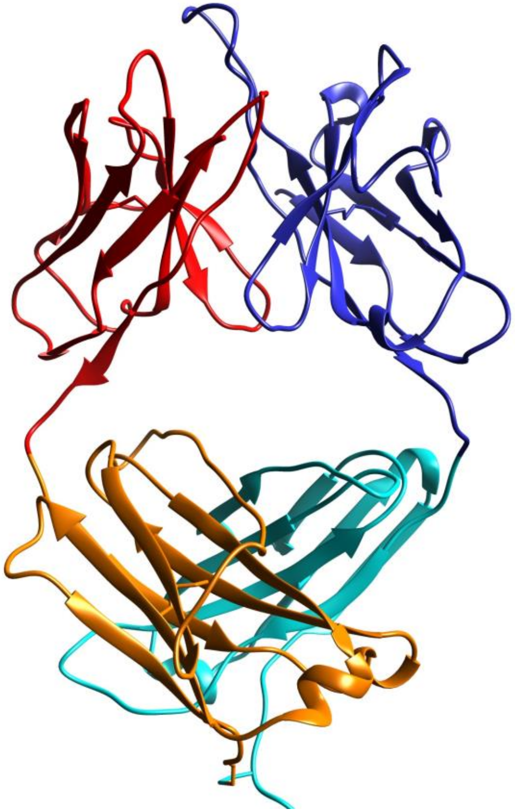
Isotopic labeling of NIST mAb Fragments in *Pichia Pastoris*

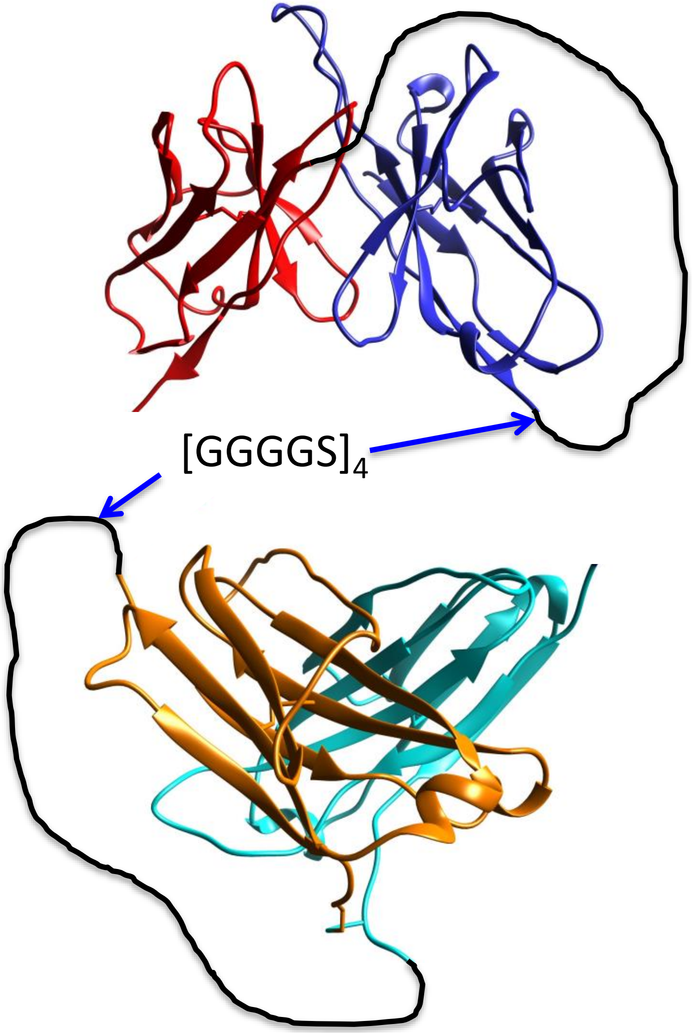
Developement and optimization of an expression protocol for the incorporation deuterium (^{2}H)

And

Methyl protonation (U-^{2}H,^{13}C,^{15}N, [^{13}CH$_3$]-Fab, Fc)

Divide and Conquer: Production of scFv and scCH1-CL for Isotopic labeling in *E. Coli*





2D $^{1}H,^{15}N$-HSQC of ^{13}C-^{15}N-NISTmAb-scFv

```
             1          2          3          4          5
    1234567890 1234567890 1234567890 1234567890 1234567890
    GSHMQVTLRE SGPALVKPTQ TLTLTCTFSG FSLSTAGMSV GWIRQPPGKA
 51 LEWLADIWWD DKKHYNPSLK DRLTISKDTS KNQVVLKVTN MDPADTATYY
101 CARDMIFNFY FDVWGQGTTV TVSSGGGGSG GGGSGGGGSG GGGSDIQMTQ
151 SPSTLSASVG DRVTITCSAS SRVGYMHWYQ QKPGKAPKLL IYDTSKLASG
201 VPSRFSGSGS GTEFTLTISS LQPDDFATYY CFQGSGYPFT FGGGTKVEIK
251 RT
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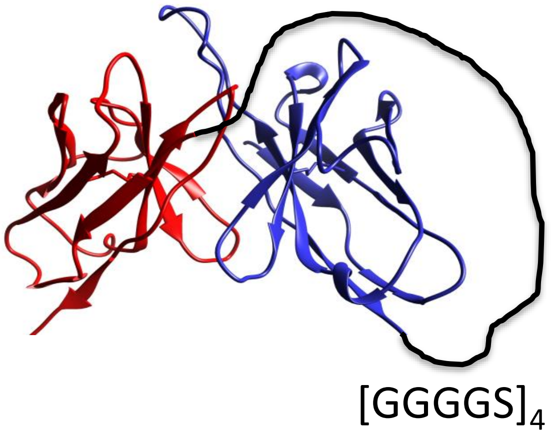
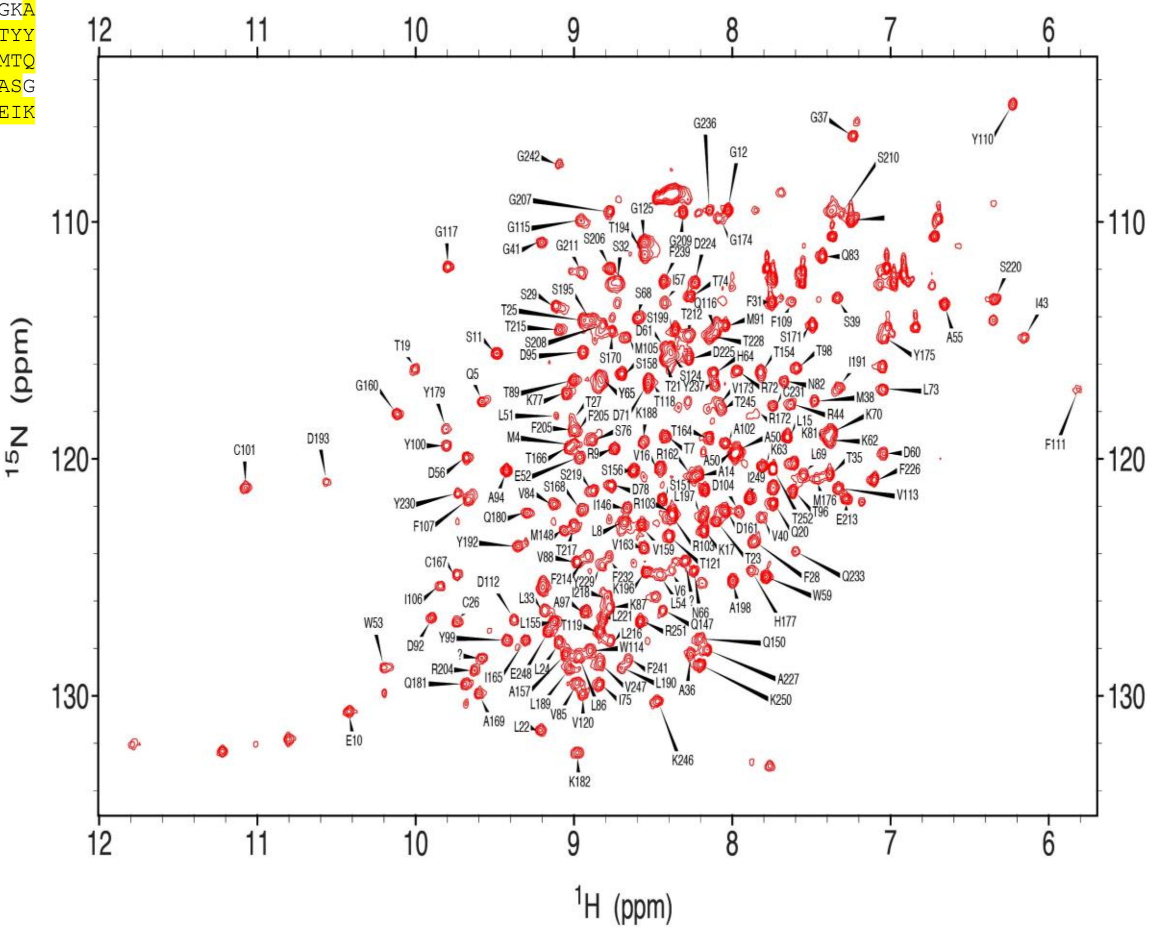
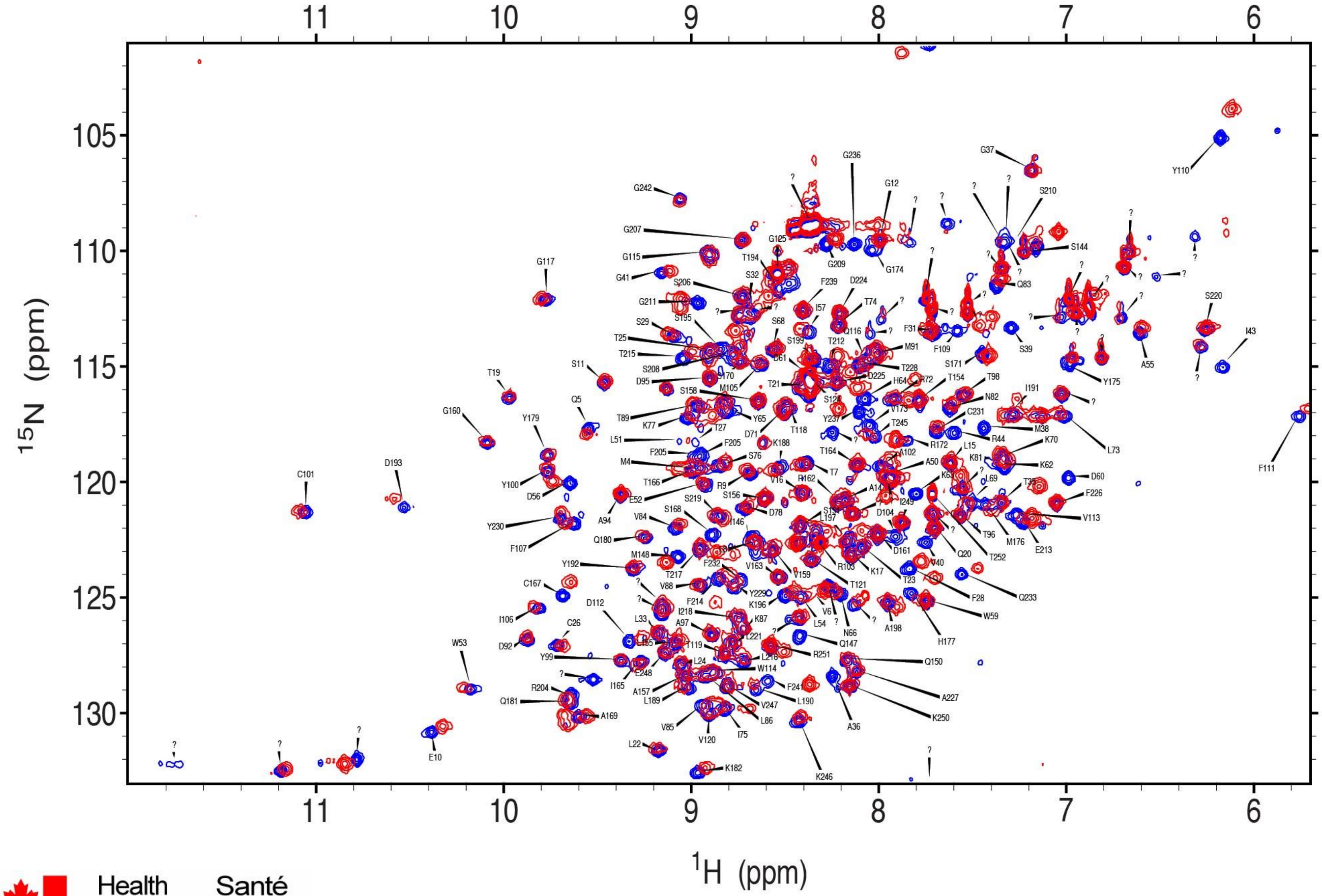
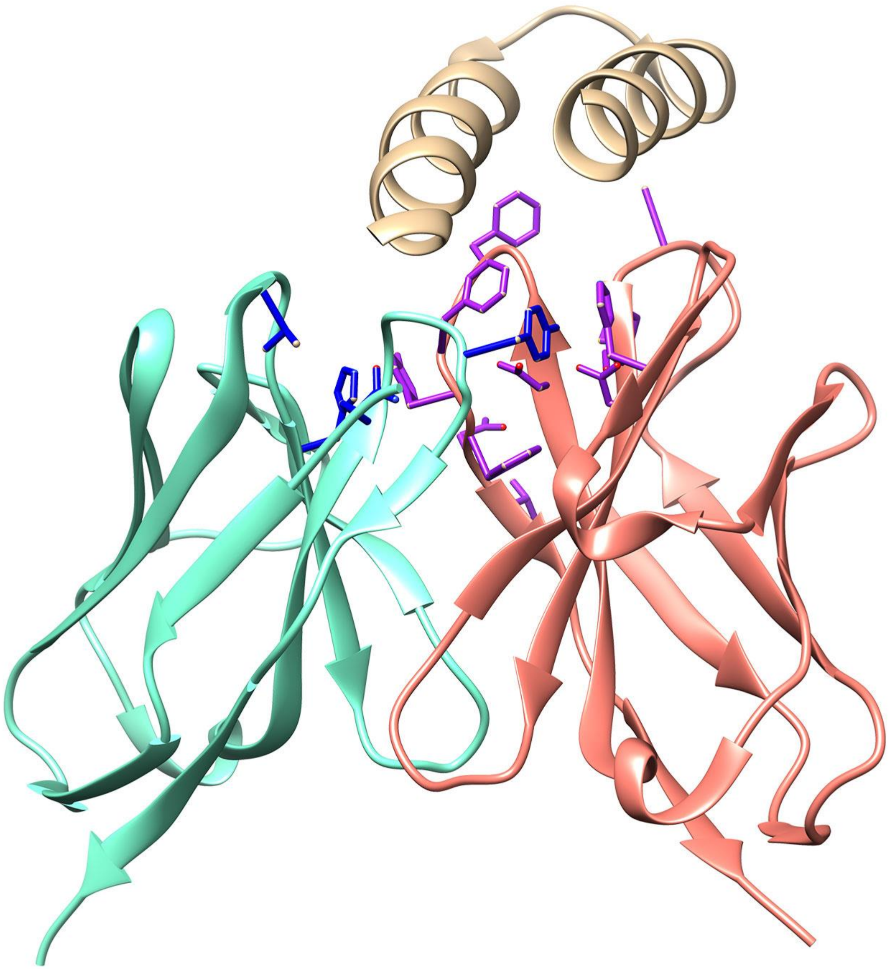




Binding of peptide derived from the RSV induces chemical shift perturbations



Mapping the largest CSPs on the Structure of the RSV Peptide Bound to the NIST-mAb



Future Directions

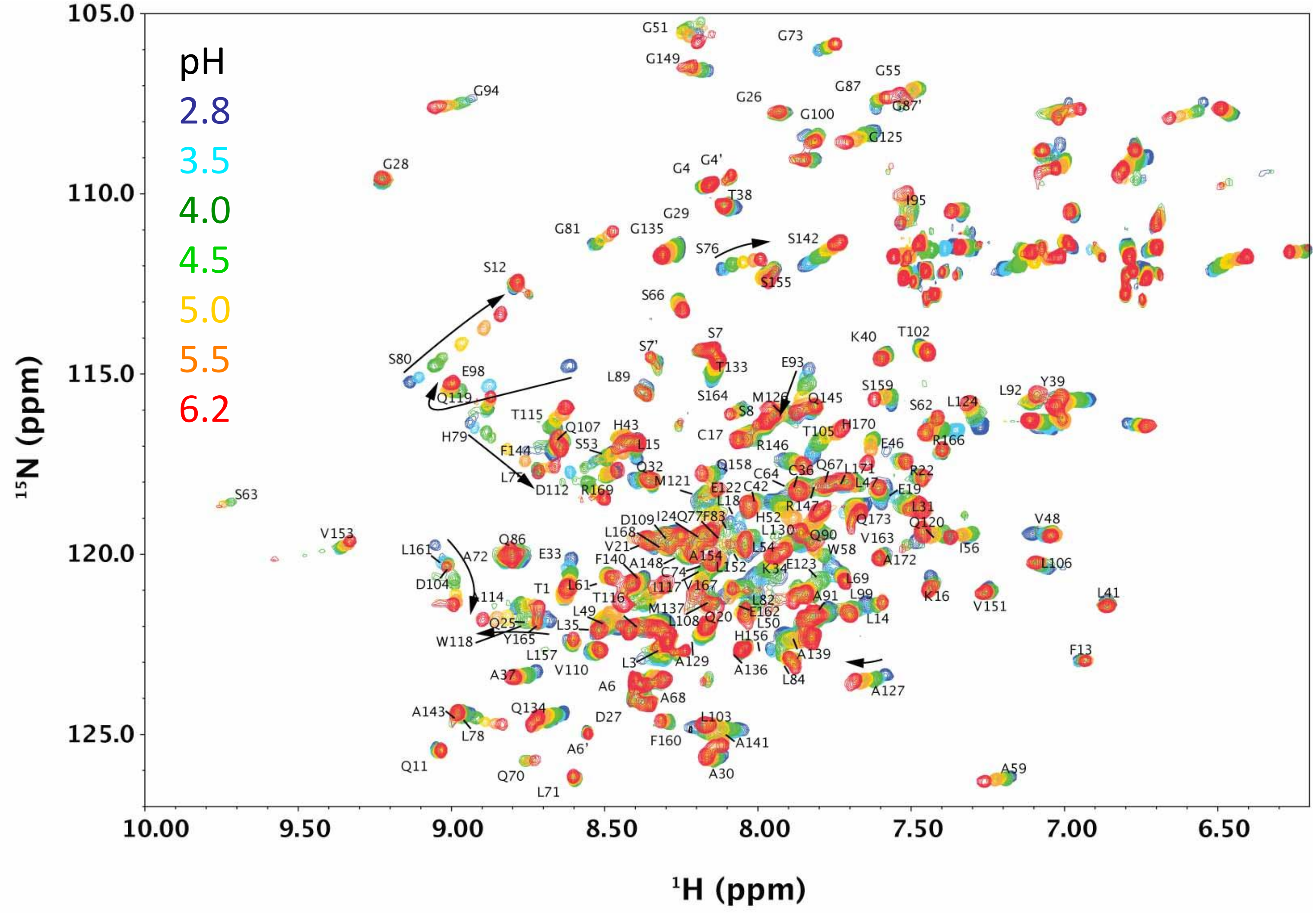
Development of the refolding and purification protocol of NISTmAb-scFab is underway (*E. coli*).

Construction and expression tests of scFab and scFv version of adalimumab, bevacizumab, infliximab, rituximab and trastuzumab in *E. coli* are completed.

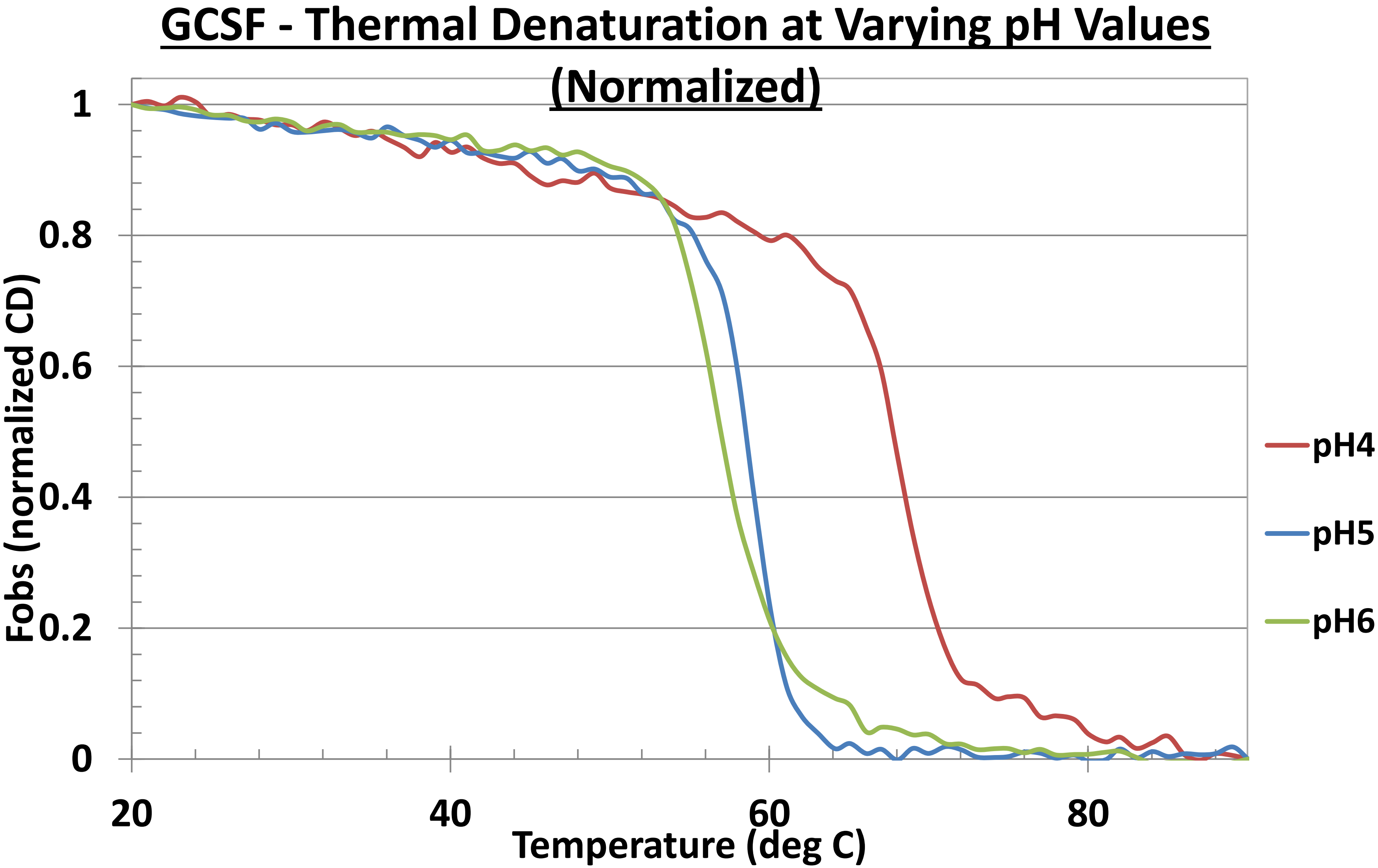
E.coli produced fragments will offer a potentially more economical avenue to study:

- Effects of excipients
- Development of a platform to study mAbs self-association

Effects of pH on chemical shifts

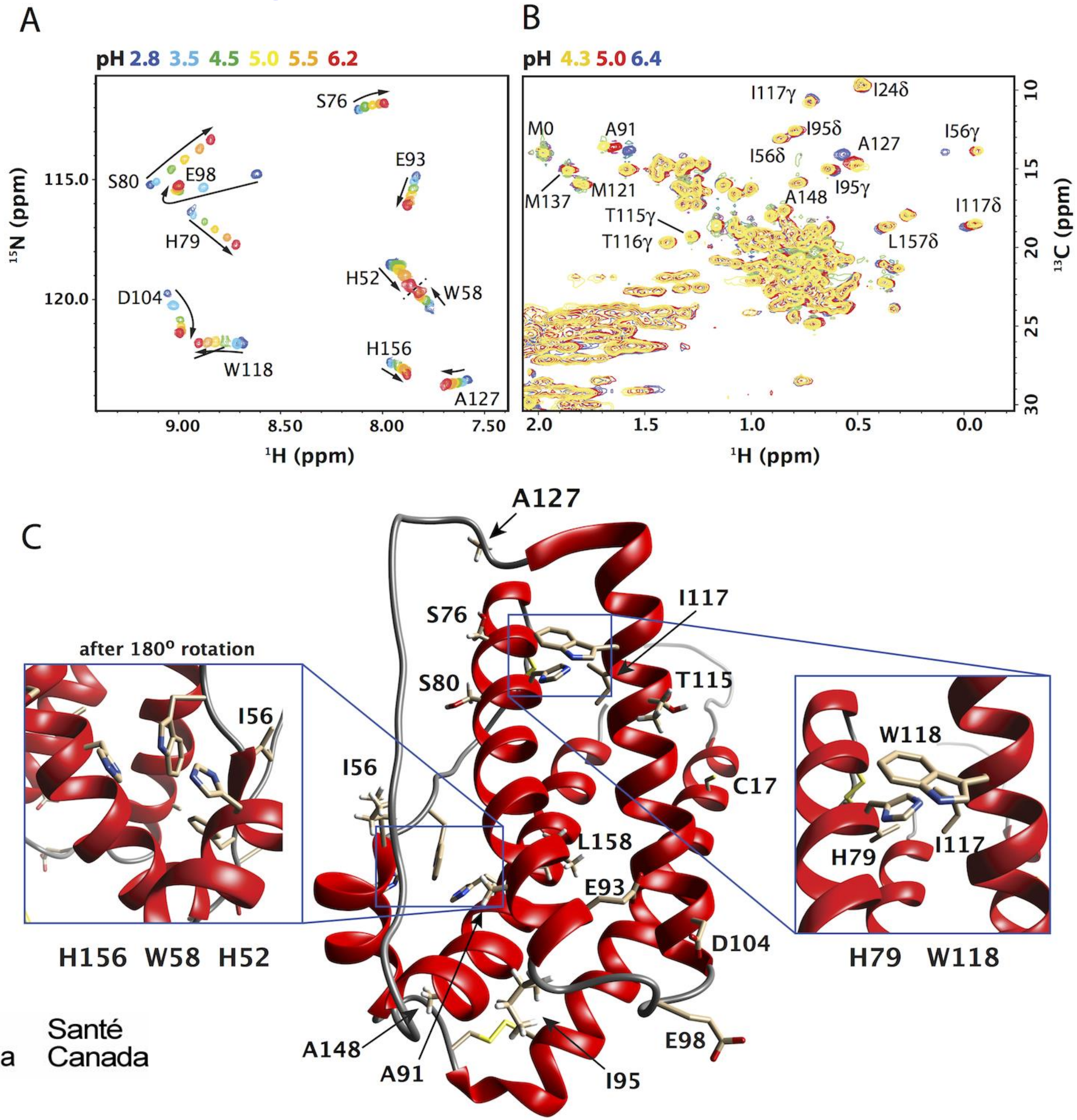


Effects of pH on Filgrastim Stability



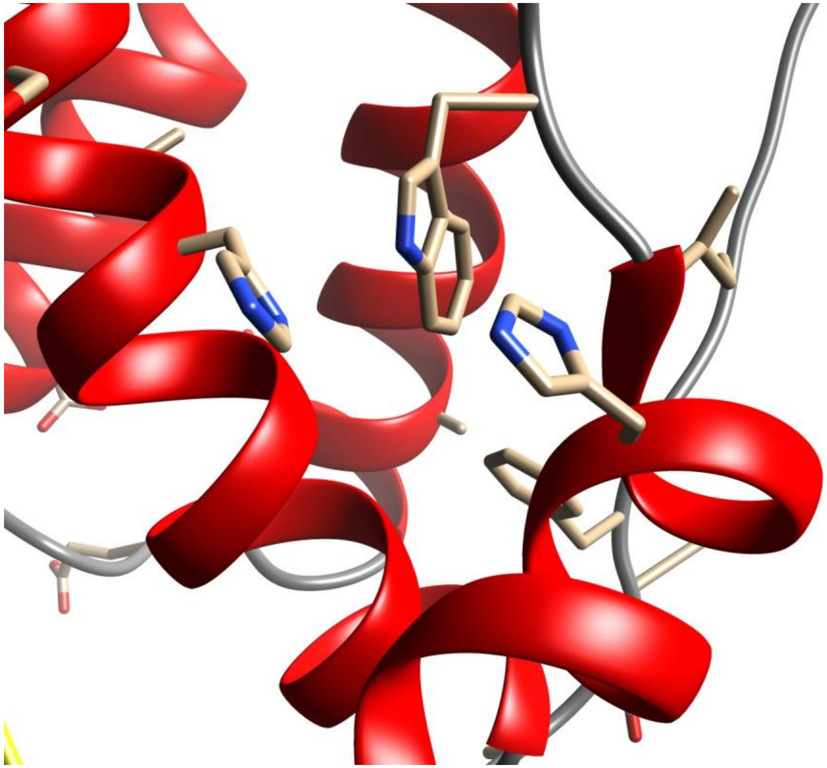
GCSF - Thermal Denaturation at Varying pH Values (Normalized)

Lowering pH: Cation-π Interaction

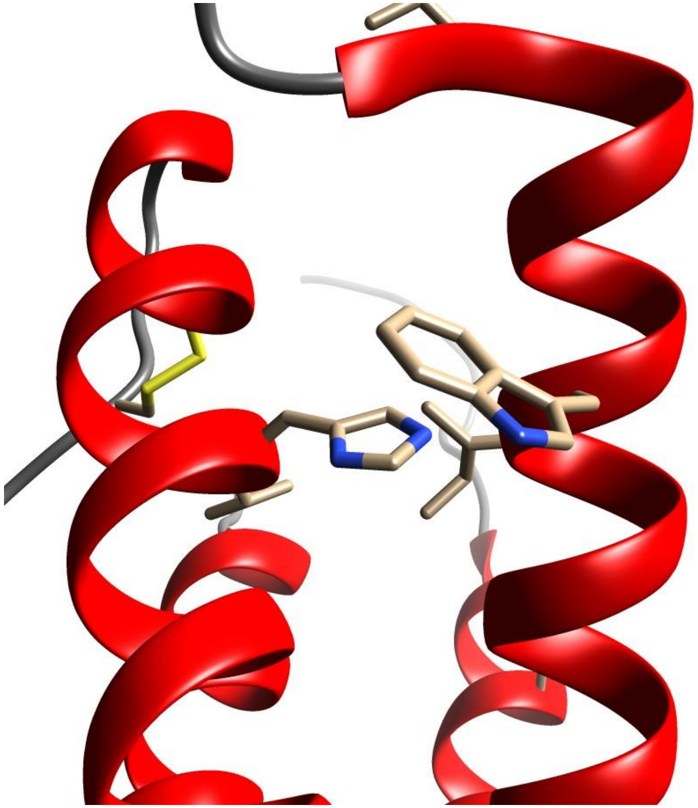


Health Canada Santé Canada

Lowering pH: Cation-π Interaction



H156 W58 H52



H79 W118

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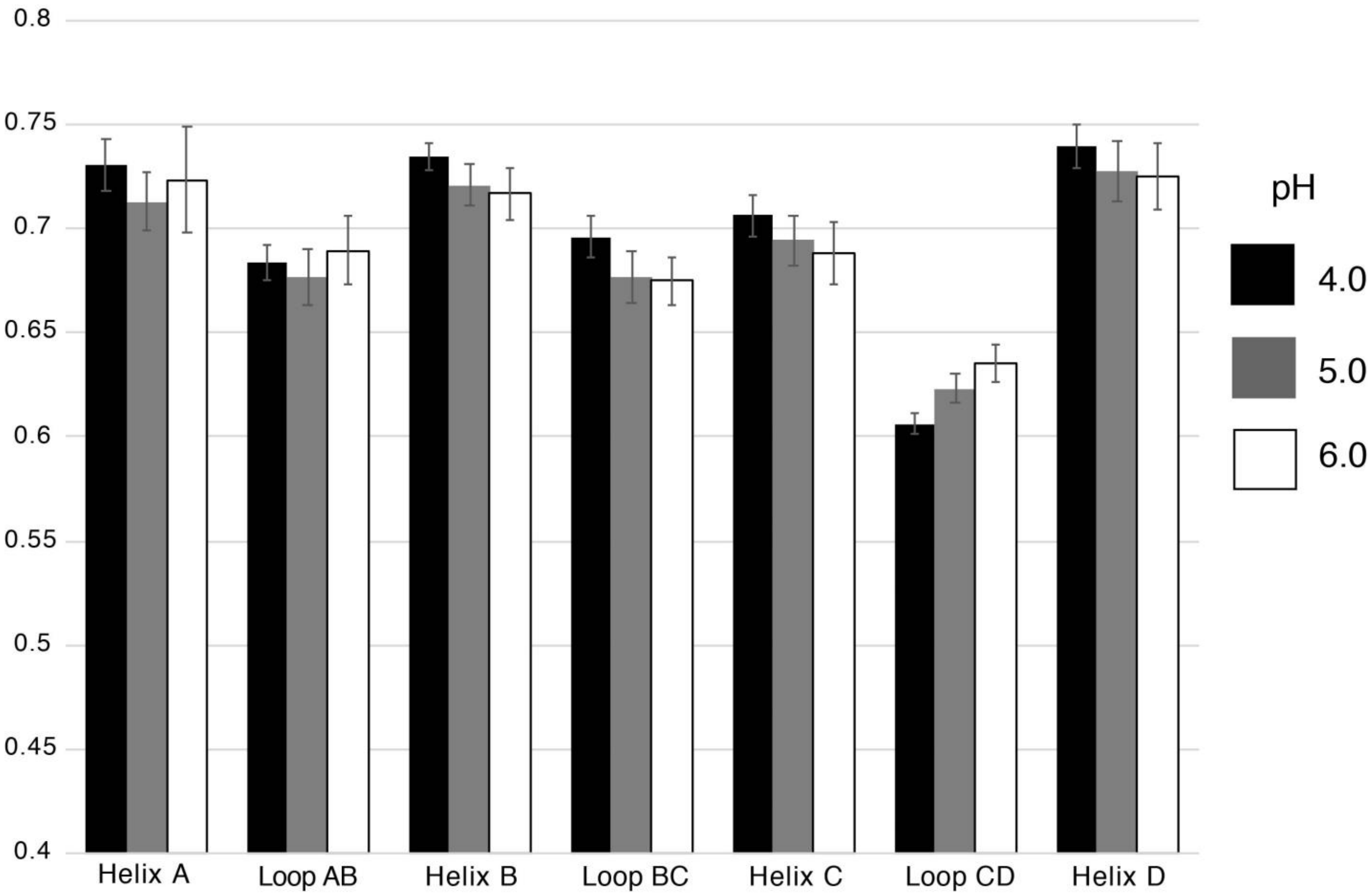
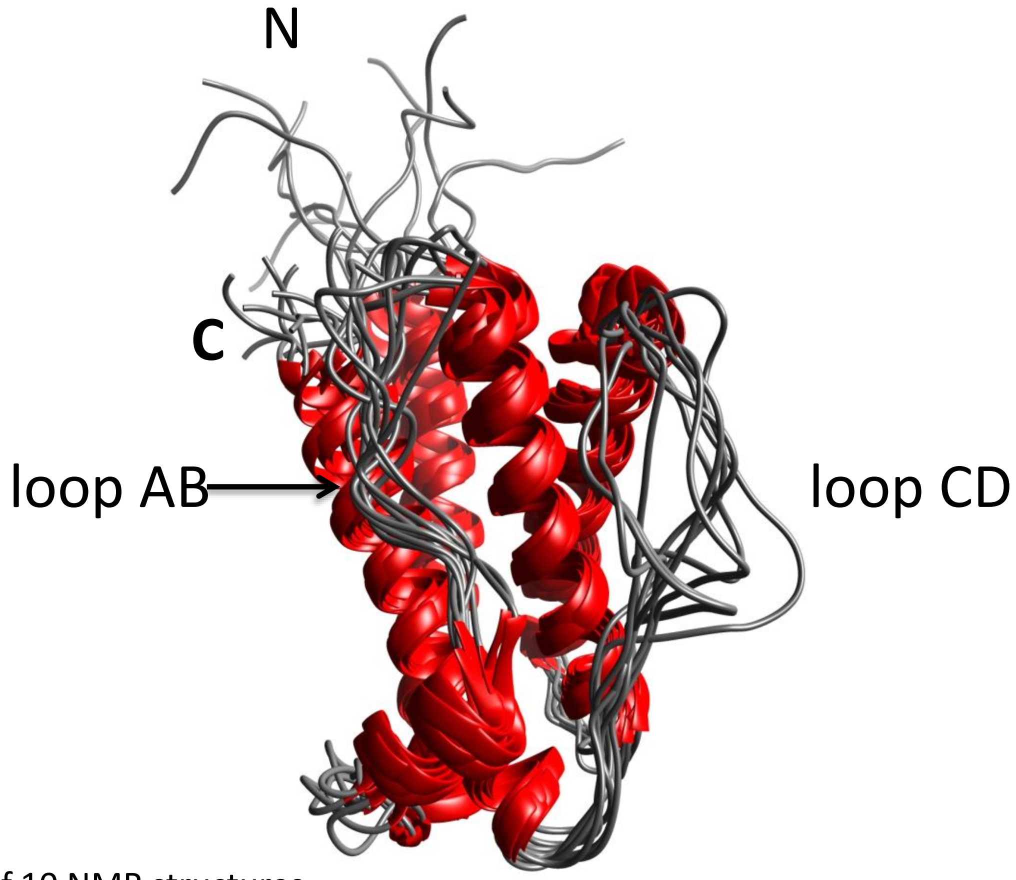
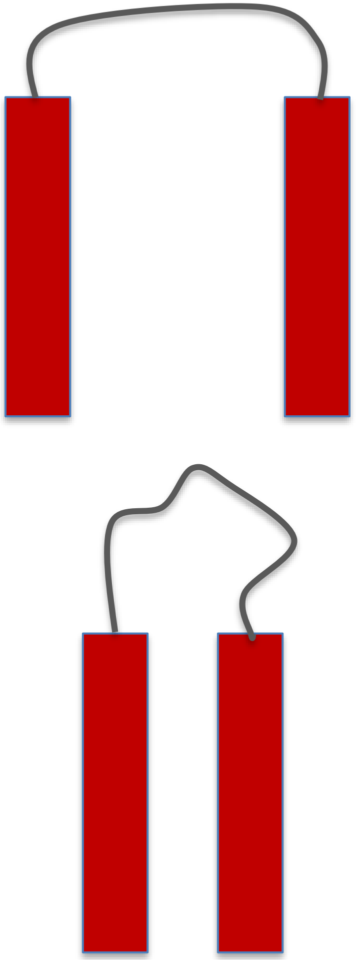
Averaging of Order Parameters Reports on Secondary Structure Elements Amplitude of Motions



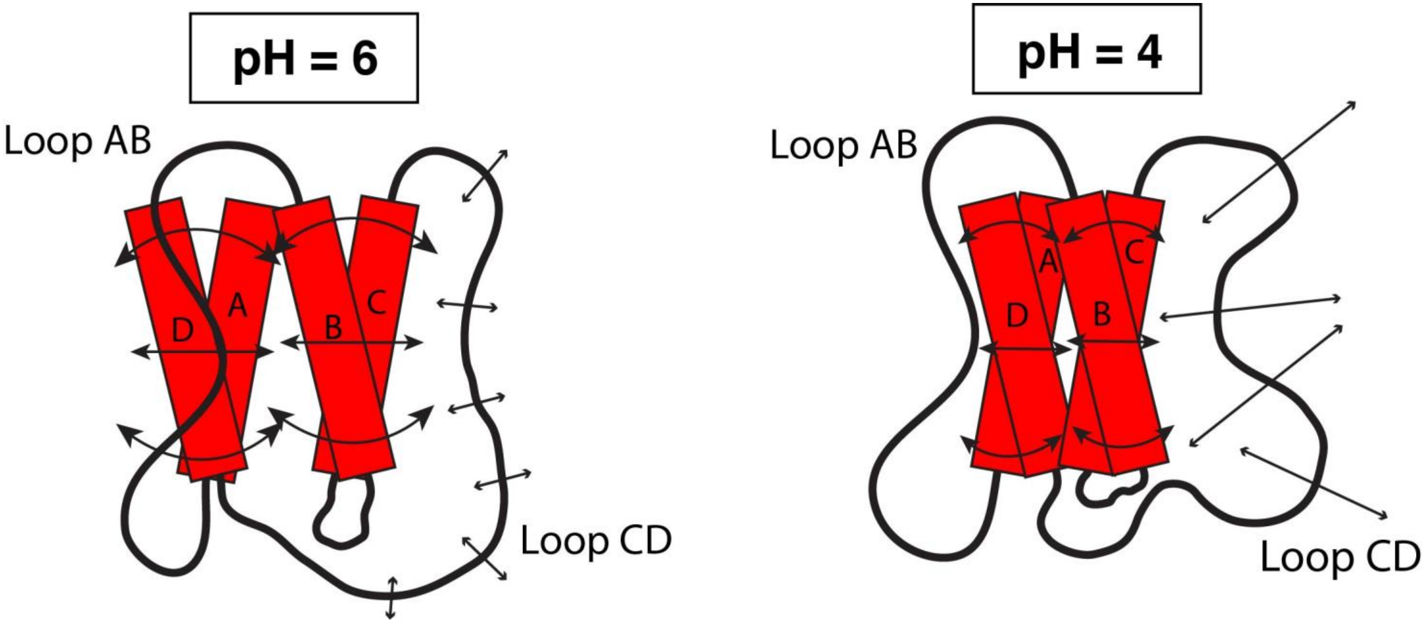
Formation of Cation-π Interaction Produces a more Compact Helix Bundle Leading to a Loosening of CD Loop



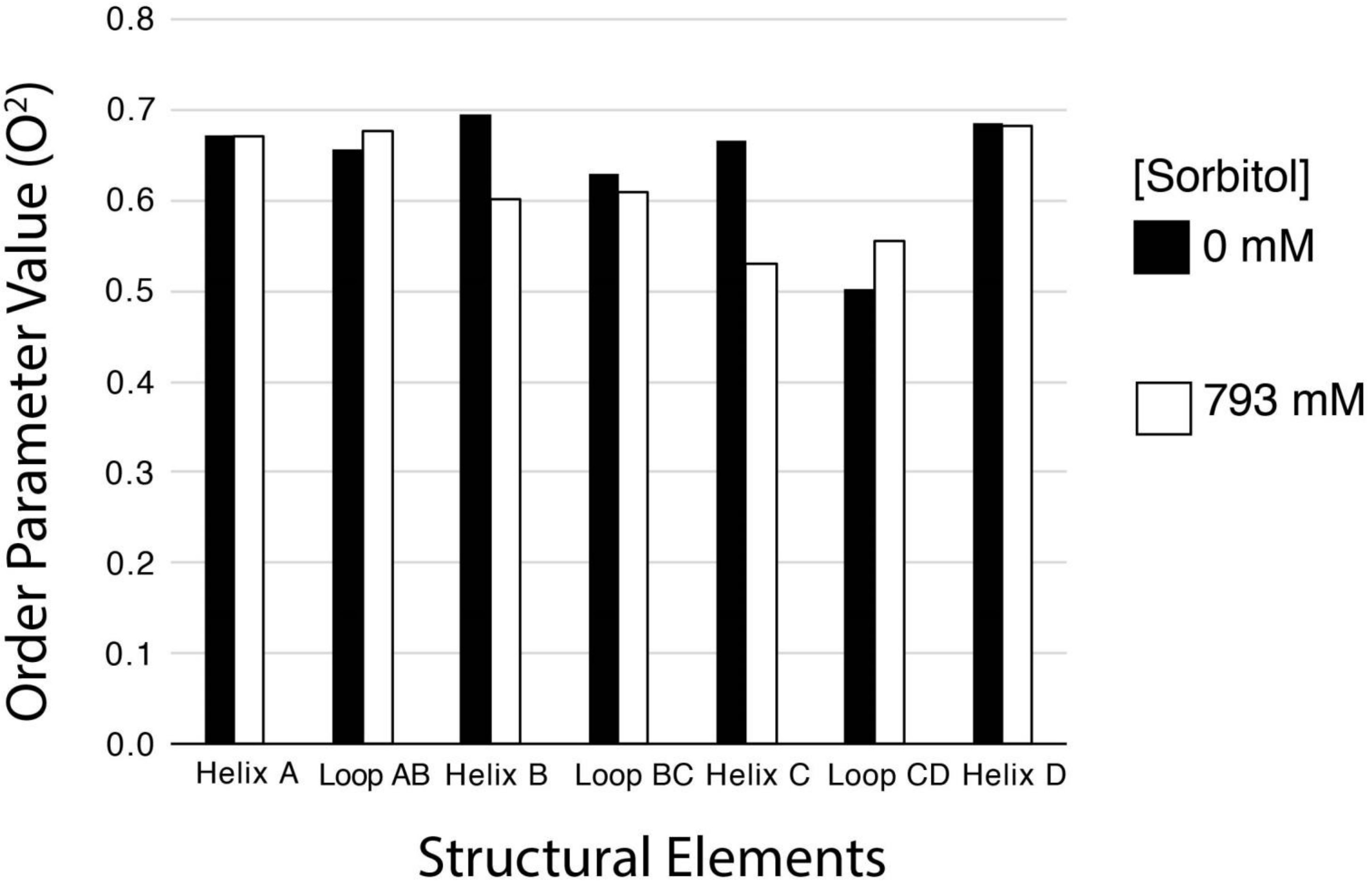


Ensemble of 10 NMR structures
1GNC.pdb
Zink et al, Biochemistry (1994) 33:8453

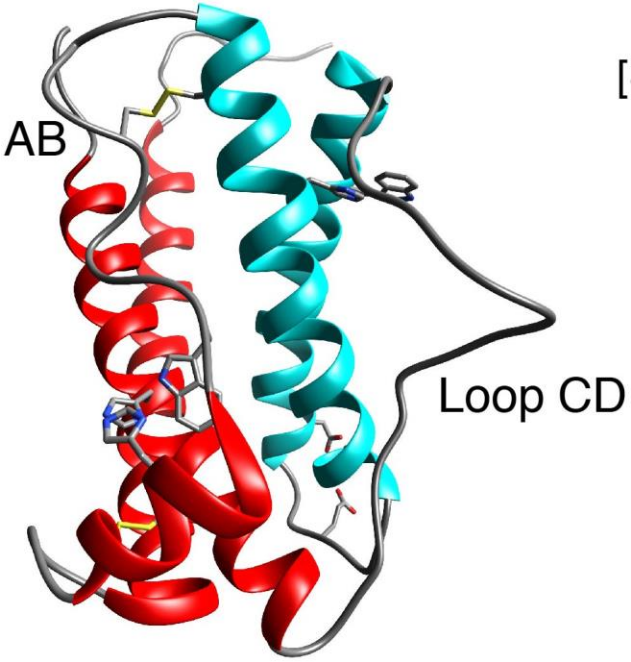
Formation of Cation-π Interaction Produces a more Compact Helix Bundle Leading to a Loosening of CD Loop

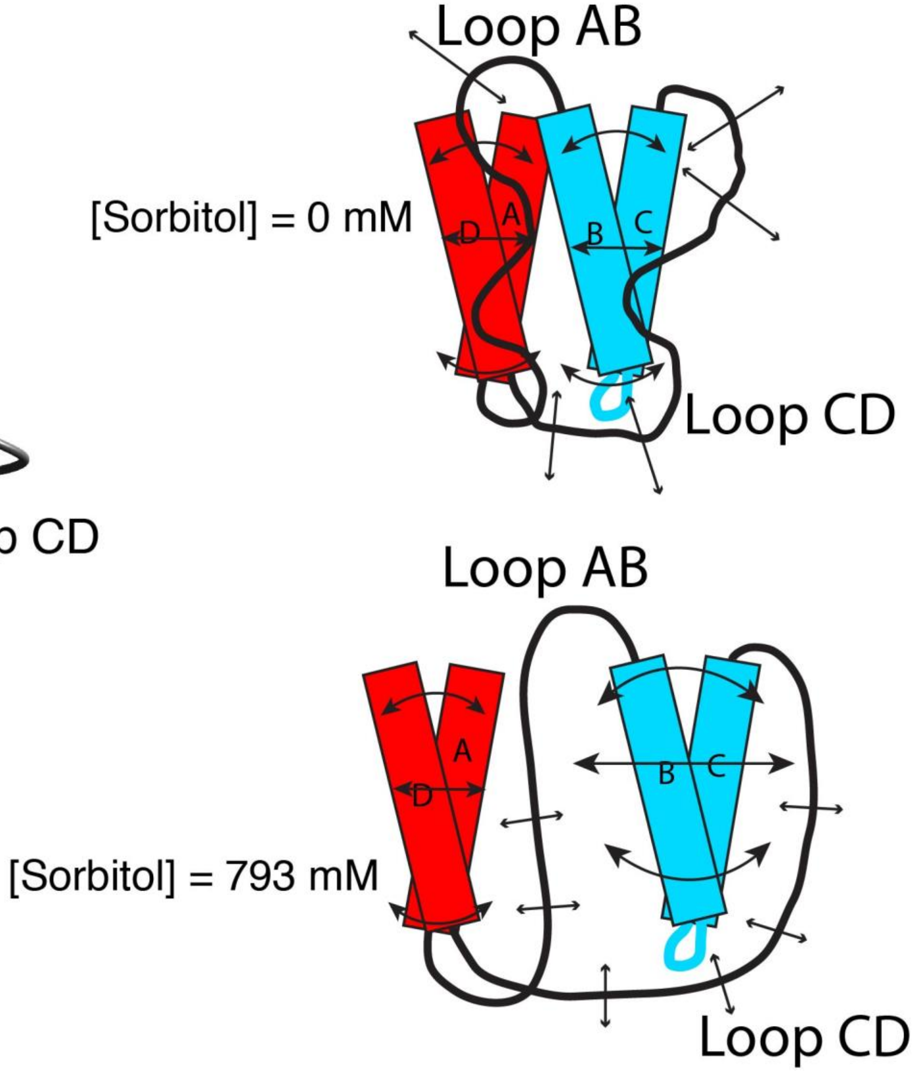


High Concentration of Sorbitol Loosens the Helix Bundle Loosening of AB and CD Loops



High Concentration of Sorbitol Loosens the Helix Bundle
Loosening of AB and CD Loops





Thank you