“Protein Stability Measurements Using Hydrogen/Deuterium Exchange, Mass Spectrometry and NMR Spectroscopy”

By Tatiana Borissova
ACKNOWLEDGEMENTS

Thank you to:

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Dr. Gaetano Montelione
Dr. Tomas Acton
Dr. James Aramini
Rong Xiao
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Chi Kent Ho
and the rest of the Montelione Laboratory
Protein Stability Determination

- Valuable information about the protein’s structure and function
- Monitor protein’s behavior in different conditions.
- Use to assay for ligand-binding, which stabilizes the folded state

\[
\begin{align*}
((P+L)_c)_H & \xrightleftharpoons[k_b]{k_{un}} (P_c)_H & \xrightleftharpoons[k_{cl}]{k_{op}} (P_{op})_H & \xrightarrow[k_{ch}]{k_{cl}} (P_{op})_D
\end{align*}
\]

Current Techniques

- Protein Stability: NMR Spectroscopy, etc.
- Ligand-binding: Nickel Column Pulldown, Yeast Two Hybrid binding assay, Protein Microarrays, Immunoprecipitation assay, etc.
- Goal: to find the most cost-, labor- and time-efficient method to assay for stability levels, structural fluctuations and ligand-binding.
NMR Spectroscopy

• Applied magnetic field causes alignment of nuclear spins
• Radiofrequency pulse is applied to tilt the nuclear magnetic vector
• Signal decay is recorded and Fourier Transformed to generate data
NMR Characteristics

- Requires large quantities of protein
- Analyses one sample at a time
- Sensitive to impurities
- Instrument is occupied for the time of experiment
- Requires a great amount of time for analysis
- Not good for analysis of large proteins
- Gives residue-specific information
MALDI-TOF Mass Spectrometry

- Protein is mixed with matrix
- Sample is shot with laser
- Ionized and vaporized protein is accelerated towards the detector
- Peak is generated for the mass/charge ratio calculated from the time of flight
- Gives non-residue specific information

http://massspec.scripps.edu/information/history/
Advantages of MS over NMR

• Small quantity of protein required
• High throughput potential
• Tolerant to impurities
• Less instrument usage time
• Less analysis time
• Easier to use
• Can analyze large proteins if use ESI/MS or digest
• Can achieve specificity through digestion
H/D exchange

• Heavier isotope
• Fast and Intermediate-exchanging amide hydrogen atoms are solvent-accessible and exchange rapidly
• Slow-exchanging amide hydrogen atoms are solvent-inaccessible and exchange slowly.
• EX1 mechanism
• H/D exchange usually through local structural fluctuations
• EX2 mechanism
• H/D exchange usually through global unfolding

\[ \Delta G_{op} = -RT \ln(K_{op}) \] for EX2

\[ K_{op} = \frac{k_{obs}}{k_{ch}}, \text{ where } k_{ch} = k_{int}[D_2O] \]
Factors Affecting H/D exchange rate

- pH
- Temperature
- Solvent accessibility
- pKa
- Neighboring amino acids

http://www.mbg.cornell.edu/nicholson/biobm631/folding/folding.html
MBP and Lambda repressor

- MBP(1-357): 40,803.8kDa. Maltose/maltodextrin system in *E.coli*
- Lambda repressor(6-85): 10,029.3kDa. Regulates lambda phage life cycle through transcriptional activation
Cloning Steps:

PCR:
- Primers used:
  - (C-MBP 1-357) F: ATCGATCGCATATCAAAAAATCGAAGAAGGTAAACTC
    R: ATCGACTCGAGAGTCTGACGACCGCTGGCG
  - (C-Lambda 6-85) F: ATCGATCACATATCAAACCATTACACAGAGCAGC
    R: ATCGACTCGAGGATTTCTCTGGCGTTGAAGGG

  94°-52°-68°-94°-57°-68°

Gel Extraction and Purification:
- run 2% Agarose gel
- cut out bands of correct size
- use Qiagen Gel Extraction kit for purification
Restriction Digest:
- use NdeI/XhoI enzymes
- incubate at 37°C overnight

Ligations:
- purify digestions using MiniElute kit
  - 2X elution with EB buffer
- ligate 1ul DNA with 1ul vector + ligation mix
- ligate overnight using a PCR machine ligations program
MBP 1-382 did not ligate

Restriction Digest of Ligations:
- incubate at 72 °C for 10 minutes
- use EcoRI/BamHI enzymes
- incubate each digestion for 1hr at 37°C
Transformations For Amplification:
- transform DNA into XL-10 Gold *E. coli* cells
- incubate for 30 minutes on ice
- heat shock at 42°C for 1 minute and incubate on ice for 5 minutes
- add SOC and incubate at 37°C for 1 hour
- add to LB/Amp plates and incubate at 37°C overnight
N-MBP 1-357 did not transform

Colony PCR:
- run colony PCR with colony PCR program
- run 2% Agarose gels
- inoculate correct construct into 2ml Superbroth/Amp

Minipreps:
- add CAM to cultures and grow a 37°C
- use Qiagen Miniprep kit protocol
  - elute twice with EB buffer
Expression Transformations For Protein Production:
- transform DNA into BL21(DE3)+Magic cells
- grow at 37°C on LB/Amp/Kan plates

Expressions (small scale):
- grow in 500ul of LB/Amp/Kan all day
- grow in 500ul of MJ9 media mix overnight
- grow in 2 mL MJ9 media mix for 3 hours
- induce with IPTG overnight at 17°C
- re-suspend in cold lysis buffer + BME and sonicate
- aliquot ‘totals’, spin and aliquot ‘supes’
- run SDS-PAGE gels

Expressions (large scale):
- grow in 40mls $^{15}$N-MJ9 media mix overnight
- grow in 1L $^{15}$N-MJ9 for 3 hours
- induce with IPTG overnight at 17°C

Solubility

Lanes 1-3: C-MBP 1-357
Lane 4: C-Lambda Standard
Lanes 5-7: N-Lambda Standard
Lanes 8-9: C-Lambda PFB 101
Protein Purification and Concentration

Ni-NTA Column Purification:
-all buffers at pH 8.0
-re-suspend in lysis buffer and lyse by sonication
-wash with wash buffer
-elute from Ni-NTA columns with elution buffer
-collect fractions, take OD, run SDS-PAGE

Gel Filtration:
-low salt buffer used: 20mM NaPi, 20mM NaAc, 100mM NaCl, 0.02% NaN₃, pH 6.3
-total volume of MBP: 37mls
-total volume of Lambda: 23mls

Concentration:
-centrifuge through concentrating columns
-final volume of MBP: 1.5mls (0.63mM)
-final volume of Lambda: 0.5mls (1.6mM)
Measuring H/D Exchange by NMR Spectroscopy

• For comparison with Mass Spectrometry
• Generates a peak for the $^{15}$N-$^1$H pair
• NMR blind to deuterium- peaks disappear over the course of exchange
• Measure the rate of disappearance by plotting $\ln$(Peak Height) vs. time
NMR Protocol #1 for Lambda repressor

- Use Varian 600
- 10°C, pH 6.3
- Mix 60ul of protein with 440ul of D₂O buffer in NMR tube for 1.68 mg/mL concentration
- Buffer: lyophilize low salt phosphate buffer (20mM NaPi, 20mM NaAc, 100mM NaCl, 0.02% NaN₃, pH 6.3), re-suspend in D₂O, adjust pH with HCl
- HSQC data collected for 14 hours
Lambda repressor NMR experiment# 1

- pH 6.3
- 10°C
- Low salt phosphate buffer
- Low peaks
- No smooth pattern of disappearance
NMR Protocol #2 for Lambda repressor

- Use Varian 500
- 20°C, pH 4.0
- Mix 1.52mg of lyophilized protein with 500ul of D₂O buffer in NMR tube for 3.04mg/mL concentration
- Buffer: 10mM ND₄Ac
- HSQC data collected for 14 hours
Lambda repressor NMR experiment #2

- pH 4.0
- 20 °C
- ND₄Ac buffer
- Disappeared quickly
- Demonstrated an exponential rate of disappearance
H/D Exchange Rate

\[ y = -0.7906x + 13.458 \]
\[ y = -0.9267x + 13.157 \]
\[ y = -0.8458x + 12.94 \]
\[ y = -0.4762x + 10.978 \]
\[ y = -0.8017x + 12.865 \]

\[ \Delta G_{\text{op}} = -RT\ln(K_{\text{op}}) = 2.07 \text{kJ/mol} \]

\[ k_{\text{ex}} \text{(average)} = 0.7682/\text{hr} = 0.0128/\text{min} \]
\[ \ln(\text{Peak Height at } t_0) = 12.68 \]
\[ \text{Peak Height (average) at } t_0 = 321,129 \]
NMR Protocol for MBP

- Use Varian 600
- 24°C, pH 6.3
- Mix 2.47mg of protein (buffer exchanged with 20mM NH₄Ac, lyophilized) with 300ul of buffer in NMR tube
- Buffer: deuterated low salt phosphate buffer (20mM NaPi, 20mM NaAc, 100mM NaCl, 0.02% NaN₃, pH 6.3)
- TROSY data collected for 15 hours
MBP NMR Experiment

- pH 6.3
- 24°C
- Low salt phosphate buffer
- TROSY
H/D Exchange Rate

\[
y = -0.17x + 12.55
\]
\[
y = -0.20x + 11.58
\]
\[
y = -0.05x + 11.33
\]
\[
y = -0.10x + 11.79
\]
\[
y = -0.09x + 11.98
\]
\[
y = -0.08x + 11.95
\]
\[
y = -0.09x + 12.63
\]

\[k_{\text{ex}}(\text{average})=0.1114/\text{hr} =0.00186/\text{min}\]
\[\ln(\text{Peak Height at } t_0)=11.97\]
\[\text{Peak Height(average) at } t_0=158,351\]
Measuring H/D Exchange by Mass Spectrometry

- Incorporation of heavier isotope of H leads to increase in mass
- Problems:
  - Back exchange
  - Drying time
Protocol #1 for MBP

24°C, pH 6.3

**H/D Exchange Reaction:** 2ul of protein + 98ul of deuterated buffer
-Buffer: 20mM NaPi, 20mM NaAc, 100mM NaCl, 0.03% NaN₃

On Ice, pH ~ 1.8

**Deuterated Protein in Matrix:** 1ul H/D ex reaction + 19ul standard in matrix
-Standard in Matrix: 3ul BSA + 122ul matrix
-Matrix: 10mg of Sinapinic Acid + 500ul of Acetonitrile + 499ul dH₂O + 1ul TFA

Spotting: spot 1ul of protein in matrix on the MALDI plate well

Room Temperature, Under the Hood

**Diluted Protein in Matrix:** 1ul protein + 19ul standard in matrix
Diluted Protein: 2ul of protein + 98ul hydrogenated buffer

Spot sample

spot sample
MBP MS Experiment #1

- pH 6.3
- Low salt phosphate buffer
- Temperature 24°C
- BSA

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<th>Delta mass</th>
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Protocol #2 for MBP

24°C, pH 6.3
H/D Exchange Reaction: 2ul of protein +
98ul of deuterated buffer
-Buffer: 20mM NaPi, 20mM NaAc, 100mM NaCl, 0.03% NaN₃

On Ice, pH ~ 1.8
Deuterated Protein in
Matrix: 1ul H/D ex reaction
+ 19ul matrix
-Matrix: 10mg Sinapinic
Acid + 500ul Acetonitrile +
499ul dH₂O + 1ul TFA

On Ice, pH 6.3
Diluted Protein in
Matrix: 1ul protein
+ 19ul matrix
-Diluted Protein: 2ul of protein + 98ul hydrogenated buffer

On Ice, pH ~ 1.8
External Standard in
Matrix I: 1ul Aldolase +
1ul Apomyoglobin +
38ul matrix
-External Standard in
Matrix II: 20ul ESM I +
20ul matrix

Previously at -20°C, kept On Ice under the hood
Spotting: spot 1ul of protein in matrix in each well on the MALDI plate
MBP MS Experiment #2

- pH 6.3
- Low salt phosphate buffer
- Temperature 24°C
- External standards
- Plate on ice

### Table

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>MBP m/z Average</th>
<th>Delta mass (m/z)</th>
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</table>
$k_{ex} = 0.246/\text{hr} = 0.0041/\text{min}$

$\ln(\Delta M_{\text{max}} - \Delta M_{t=0}) = 5.57$

$\Delta M_{\text{max}} - \Delta M_{t=0} = 261.73$

$\Delta M_{t=0} = 82.27$

<table>
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<tr>
<th>Time (x)</th>
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</table>
Protocol #1 for Lambda repressor

24°C, pH 7.0

H/D Exchange Reaction: 2ul of protein + 98ul of deuterated buffer
-Buffer: 20mM NaPi, 20mM NaAc, 100mM NaCl, 0.03% NaN₃

On Ice, pH ~ 1.8
Deuterated Protein in Matrix: 1ul H/D ex reaction + 19ul standard in matrix
-Standard in Matrix: 3ul Apomyoglobin + 122ul matrix
-Matrix: 10mg of Sinapinic Acid + 500ul of Acetonitrile + 499ul dH₂O + 1ul TFA

Room Temperature, Under the Hood
Spotting: spot 1ul of protein in matrix on MALDI plate well

Spot sample

On Ice, pH 7.0
Diluted Protein in Matrix: 1ul protein + 19ul standard in matrix
Diluted Protein: 2ul of protein + 98ul hydrogenated buffer

Spot sample
Lambda Repressor MS Experiment #1

- pH 7.0
- Low salt phosphate buffer
- Temperature 24°C
- Apomyoglobin

### Lambda H/D exchange, pH 7.0

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<th>Delta m/z</th>
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Protocol #2 for Lambda repressor

22°C, pH 4.0

H/D Exchange Reaction: 2ul of protein + 98ul of deuterated buffer
-Buffer: 10mM ND₄Ac

On Ice, pH ~ 1.8
Deuterated Protein in Matrix: 1ul H/D ex reaction + 19ul matrix
-Matrix: 10mg Sinapinic Acid + 500ul Acetonitrile + 499ul dH₂O + 1ul TFA

On Ice, pH 4.0
Diluted Protein in Matrix: 1ul protein + 19ul matrix
-Diluted Protein: 2ul of protein + 98ul hydrogenated buffer

On Ice, pH ~ 1.8
External Standard in Matrix I: 1ul Insulin + 2ul Apomyoglobin + 17ul matrix
External Standard in Matrix II: 10ul ESM I + 10ul matrix

Previously at -20°C, kept On Ice under the hood
Spotting: spot 1ul of protein in matrix in each well on the MALDI plate
Lambda Repressor MS Experiment #2

- pH 4.0
- ND$_4$Ac
- Temperature 22°C
- External standards
- Plate on ice

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H/D Exchange Rate

\[ \text{k}_{\text{ex}} = 0.666/\text{hr} = 0.0111/\text{min} \]
\[ \ln(\Delta M_{\text{max}} - \Delta M_{t=0}) = 3.60 \]
\[ \Delta M_{\text{max}} - \Delta M_{t=0} = 36.65 \]
\[ \Delta M_{t=0} = 50.34 \]
\[ \Delta G_{\text{op}} = -RT\ln(K_{\text{op}}) = 2.44\text{kJ/mol} \]

**Table:**

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<th>ln(dMmax-dM)</th>
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Lambda Repressor MS and NMR

**Lambda H/D exchange, pH 4.0**

**H/D Exchange Peaks**

\[ k_{ex} = 0.666/\text{hr} = 0.0111/\text{min} \]

\[ \ln(\Delta M_{\text{max}} - \Delta M_{t=0}) = 3.60 \]

\[ \Delta M_{\text{max}} - \Delta M_{t=0} = 36.65 \]

\[ \Delta M_{t=0} = 50.34 \]

\[ \Delta G_{op} = 2.44 \text{kJ/mol} \]

\[ k_{ex}(\text{average}) = 0.7682/\text{hr} = 0.0128/\text{min} \]

\[ \ln(\text{Peak Height at } t_0) = 12.68 \]

\[ \text{Peak Height (average) at } t_0 = 321,129 \]

\[ \Delta G_{op} = 2.07 \text{kJ/mol} \]
MBP MS and NMR

\[
k_{ex} = 0.246/\text{hr} = 0.0041/\text{min}
\]
\[
\ln(\Delta M_{\text{max}} - \Delta M_{t=0}) = 5.57
\]
\[
\Delta M_{\text{max}} - \Delta M_{t=0} = 261.73
\]
\[
\Delta M_{t=0} = 82.27
\]

\[
k_{ex} \text{ (average)} = 0.1114/\text{hr} = 0.00186/\text{min}
\]
\[
\ln(\text{Peak Height at } t_0) = 11.97
\]
\[
\text{Peak Height (average) at } t_0 = 158,351
\]
If I Had More Time…

• Optimize the Mass Spectrometry method
• Perform denaturant concentration vs. time H/D experiments
• Perform ligand-binding experiments
References


etc.