H/D Exchange and Limited Proteolysis

Mass spectrometry facility
CABM
Room 112
Introduction

- Majority of protein structures are solved using NMR or X-ray crystallography
- Unstructured regions of the protein affect NMR results and crystal formation
- Mass spectrometry offers potentially rapid and inexpensive methods to determine these unstructured regions
- This information can be helpful for construct optimization or characterization of intrinsically unfolded proteins
H/D Exchange

Amide hydrogen exchange rate is affected by:
- pH
- temperature
- sequence
- structure
Sample Preparation

Protein sample
5 μl 10 mg/ml

Buffer (D₂O)
15 μl TBS

Variables: temperature, pH, time points

~pH 2.5, mild denaturing condition
on dry ice or stored in -80°C

Lesley et al, Protein Sci. 2004, 13: 3187-3199
Sample Analysis

Reduce Back Exchange:
- pH ~3, on ice
- Fast digestion and HPLC
Optimization of Sample Loading: -Digestion

Injection flowrate=500 μl/min

Injection flowrate=250 μl/min

Injection flowrate=150 μl/min

Intact protein
Optimization of Sample Loading:
- Back-exchange

Cyto-C, 100 Sec Labeling

# deuteriums incorporated

Loading time (minutes)
Calculation of Deuterium Level

Average M/Z = 576.13

\[ \Delta M = (576.7 - 576.13) \times Z = 1.14 \]

\[ \Delta M_{100} = (576.9 - 576.13) \times Z = 1.58 \]

*Deuterium level = \( \frac{\Delta M}{\Delta M_{100}} \) = 72.1%

Test Case: WR33

- 190 residues; NMR assignments on ~ 5 mg

- Very laborious to determine assignments because of crowded spectra of unfolded regions

- H/D exchange by MS at ExSAR confirmed same disordered region using μg of sample; much faster

- These data used to optimize construct, several constructs screened, solved NMR structure of WR33 (1-115) (Tejero, Hunt, Weber, Montelione, unpublished)
HD Exchange Experiment Procedure

- **Experiment 1:** unlabeled protein,
  - Optimize experimental conditions
    - Digestion time,
    - HPLC
  - Peptide selection:
    - Maximum sequence coverage
    - Reproducible

- **Experiment 2:** D-labeling, time points

- **Experiment 3:** D-labeling, maximal labeling as control
WR33: Choice of Peptides

MGHHHHHSHMAAAAGFNWDDADVKKRWDAFTKFGAATATEMTGKNFDKWLKDAGVLDN

KAITGTMTGIAFSKVTGPKKKATFDETKKVLAFVAEDRARQSKKPQDELDAITEKLAK

LEAPSVGAAKANAAGVYSRLTDHTKYTGAHKERFDAEGKKGKSGRATTTENTGYVGA

YKNKDSYDKTHGK

Peptide identified
Peptides picked
WR33 H/D Exchange Data Agrees Well with ExSAR and NMR Data
Limited Proteolysis

- The accessibility of a folded protein to proteases is highly affected by the structure.
- By proteolysis under native conditions, tightly folded core and digested pieces from unstructured region can be observed by mass spectrometry.

Intact protein  Protease  Resistant core  Fragments
Limited Proteolysis of WR33: Resistant Core

MALDI-TOF analysis, linear mode

Tyrpsin on ice

0 min

5 min

10 min

30 min

MALDI-TOF analysis, linear mode
Limited Proteolysis of WR33: Peptides

WR33-Trypsin 10 min, on ice, peptides
MS/MS allows definitive peptide assignment

MSMS of 3063.5671SGRADTTE\text{NTGYVGAYKNKDSYDKTHGK}

\begin{figure}[h!]
\centering
\includegraphics[width=\textwidth]{mass_spectrum.png}
\caption{Mass spectrum of peptide sequence NTGYVGAYKNKDSYDKTHGK.}
\end{figure}
Different Proteases Offer Complementary Information

WR-33 + Chymotrypsin (10 min, 0°C)

WR-33 + protease K (10 min, 0°C)
WR33 Limited proteolysis data agrees with H/D Exchange and NMR Data
Summary for Limited Proteolysis

- Large fragments were observed using MALDI-TOF in linear mode.
- Small fragments were observed using MALDI-TOF in reflector mode and MS/MS was conducted on certain peaks for definitive assignment.
- Disordered and tightly packed regions can be distinguished from unstructured part.
- Limited proteolysis data agree well with H/D exchange data and may be used for corroboration.
Summary for HD/Exchange and Limited Proteolysis

- Both H/D exchange and limited proteolysis can be used for distinguishing structured and unstructured region of target proteins.
- Both techniques can be used for identification of inter-domain linkers and domain boundaries.
- H/D exchange were routinely used for target optimization (one target/week on average).
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