Overview

- **MALDI-TOF MS**
  - Protein modifications
  - Peptide mass fingerprinting
- **Electrospray MS**
  - Analysis of intact proteins
  - Molecular weight calculations
  - Max Entropy for MW estimation
- **Peptide analysis**
  - Purity - ESI-MS is a revelation
- **Integration of MS with LC and CE**
  - Multidimensional LC of peptides
- **Tandem MS**
  - Identifying peptide amino acid sequences
Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

- Advantages of MALDI-TOF
  - More tolerant to common buffers than ESI
  - High degree of sensitivity, moderate mass accuracy, and mass resolution
  - High mass compounds, i.e. proteins, PEG...

- Common Applications of MALDI-TOF
  - Masses of large proteins and other compounds
  - Enzymatic digestion profiles of proteins to establish their identity
  - Peptide sequencing (TOF-TOF)

Factors from conventional experiments that impact MALDI-TOF analysis

- Tolerance of buffers/chemicals used in sample preparation
  - NaCl up to 150 mM
  - Urea up to 2-3 M (carbamoylation can occur!)
  - Guanidinium-HCl up to 2 M

- Tolerance of detergents
  - SDS up to 0.05%

- Staining Protocols
  - Whole proteins form adducts with Coomassie
  - Silver staining modifies selected peptides
Matrix-Assisted Laser Desorption Ionization (MALDI)

Flight tube and drift region to measure the time-of-flight (TOF)

Accelerating pulse

Chemically modifying an antibody

Scheme 1

\[
\text{PTX-2'-OH} + \text{Succinic Anhydride} \rightarrow \text{PTX-2'-O}_2\text{CCH}_2\text{CH}_2\text{CO}_2\text{H}
\]

PTX-SX

Scheme 2

\[
\text{MAb} + \text{PTX-SX} \xrightarrow{\text{NHS/EEDQ, DMF}} \text{NH-PTX}
\]

PTXMAb

Ahmad Safavy
Structure of modified antibody

Modification of an antibody by MALDI-TOF

Unreacted Ab

Reacted Ab

\[ \Delta = 2925 \text{ Da} \]
A mass spectrum of peptides from a tryptic digest

Isotope profile of individual peptide ion
Increased sensitivity in reflector vs. linear mode

Benefit of removing salt from tryptic digest

S. Barnes-UAB 1/24/06
Peptide mass fingerprinting

- This method has been developed because of the availability of predicted protein sequences from genome sequencing.
- Proteins do not have to have been previously sequenced - only that the open reading frame in the gene is known - the rest is a virtual exercise in the hands of statisticians, bioinformaticists and computers.
**Choice of peptidase**

- Analogous to DNA restriction enzymes
- Tryptic peptide fingerprinting may identify several highly related protein candidates (e.g., actins)
- Inspection of the sequences may reveal that there is a difference at one residue that distinguishes between two candidates.
- If for instance it is a glutamate, then use of Glu-C or V8-protease may enable the two proteins to be correctly identified
- INSPECT sequences carefully

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**Proteolytic enzymes used to hydrolyze proteins**

The choice of enzyme largely depends on the nature of the amino acid sequence and the specific issue that is being addressed

- Trypsin - cleaves at arginine and lysine residues
- Chymotrypsin - cleaves hydrophobic residues
- Arg-C - cleaves at arginine residues
- Glu-C - cleaves at glutamic acid residues
- Lys-C - cleaves at lysine residues
- V8-protease - cleaves at glutamic acid residues
- Pepsin - cleaves randomly, but at acid pH

See http://www.abrf.org/JBT/1998/September98/sep98m_r.html
Searching databases with peptide masses to identify proteins

Best site is at www.matrixscience.com

The program (MASCOT) can search the MSDB or NCBI databases using a set of tryptic peptide masses, or the fragment ions (specified or unspecified) of peptides

Presents the expected set of tryptic peptides for each matched protein

MALDI-TOF mass spectrum of tryptic digest of p22 band purified by 6xHis-tag
### Probability Based Mowse Score

Score is $-10 \times \log(P)$, where $P$ is the probability that the observed match is a random event.

Protein scores greater than 71 are significant ($p<0.05$).

<table>
<thead>
<tr>
<th>Accession</th>
<th>Mass</th>
<th>Score</th>
<th>Description</th>
</tr>
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<tr>
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<td>548939</td>
<td>20840</td>
<td>108</td>
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<td>gi</td>
<td>13384624</td>
<td>46931</td>
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### MASCOT SEARCH SUMMARY

1. gi|548939 Mass: 20840 Score: 108 FKBP-TYPE PEPTIDYL-PROLYL CIS-TRANS ISOMERASE SLYD (PPIASE) (ROTAMA)

<table>
<thead>
<tr>
<th>Observed</th>
<th>Mr(expt)</th>
<th>Mr(calc)</th>
<th>Delta</th>
<th>Start</th>
<th>End</th>
<th>Miss</th>
<th>Peptide</th>
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<tr>
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<td>3857.71</td>
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<td>FLAETDQGPVPVEITAVEDDHVVVDGHNHMLAQGQLNKL</td>
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No match to: 1262.47, 2343.88

2. gi|13384624 Mass: 46931 Score: 45 myocyte enhancer factor 2C (Mus musculus)

<table>
<thead>
<tr>
<th>Observed</th>
<th>Mr(expt)</th>
<th>Mr(calc)</th>
<th>Delta</th>
<th>Start</th>
<th>End</th>
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No match to: 1262.47, 2343.88

3. gi|5257384 Mass: 43424 Score: 44 (AF137308) phytochrome B (Lolium perenne)

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<th>Mr(expt)</th>
<th>Mr(calc)</th>
<th>Delta</th>
<th>Start</th>
<th>End</th>
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<th>Peptide</th>
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No match to: 1262.47, 2343.88

4. gi|4505147 Mass: 50305 Score: 44 MADS box transcription enhancer factor 2, polypeptide C (myocyte enhan

<table>
<thead>
<tr>
<th>Observed</th>
<th>Mr(expt)</th>
<th>Mr(calc)</th>
<th>Delta</th>
<th>Start</th>
<th>End</th>
<th>Miss</th>
<th>Peptide</th>
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<td>220</td>
<td>0</td>
<td>NSIMSPGVTHRPPSAAGNTGGLMGDLTSGAGTSAGNGYDNPR</td>
</tr>
</tbody>
</table>

No match to: 1262.47, 2343.88

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**E. coli**: FKBP-TYPE PEPTIDYL-PROLYL CIS TRANS ISOMERASE

Nominal mass of protein (Mr): 20840

1 MKVAKDLVVS LAYQVRTEDG VLVDSPVSA PLDYLHGHGS
41 LIGSLETAE GHEVGDKFDV AVGANDAYQY YDENLVQVRP
81 KDVFDMGVEQ LGVMRFLAET DQGPVFVEIT AVEDDHVVVD
121 GNHMLAGQNL KFNVEVVAIR EATEEELAHG HVHGAHDDHH
161 DHDHDGCCGG HGHDHGHEHG GEGCCHGKGN GGCCH

Tryptic fragments detected by MALDI-TOF-MS

132–140 FNVEVVAIR
6–16 DLVVSLAYQVR
58–78 FDVAVGANDAYQYDENLVQ
96–131 FLAETDQGPVFVEITAVEDHVVDGNHMLAGQNLK

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**Other web sites for peptide analysis**

- [http://prowl.rockefeller.edu/](http://prowl.rockefeller.edu/) – Choose ProFound
- [http://prospector.ucsf.edu/](http://prospector.ucsf.edu/) – Choose MS-fit

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Further information on identified protein

- Take the protein identifier number:
  - For neutrophil calgranulin A it is gi|28782
  - Under Entrez, paste in the gi number
  - A link to the protein will appear
  - Click on Blink - this is similar to BLAST, but better
  - Select 3D-structures on this page to get Protein Data Base record(s) of crystal structure data of the nearest protein - this will yield 1XK4
  - Go to Structure (top of web page) and enter 1XK4 and click on its icon on the next page
  - To view a 3D-image of the protein, first download Cn3D from the NCBI site

Examples for homework (due Jan 31)

- Identify the following proteins from these MALDI ions (corrected for isotope effects):
  - 910.46, 1350.81, 1515.66, 1632.87, 1800.92, 1853.96 (human)
  - 965.46, 998.56, 1001.45, 1068.48, 1581.81, 1677.96 (rat)
  - 937.52, 972.49, 1049.56, 1209.64, 1508.70, 1844.89 (mouse)

- Set the number of tryptic cuts to 0 and try varying the mass accuracy from 0.02 to 1.0 Da. How does this alter the MOWSE score?
How to represent the mass of compound?

- At low mass resolution (where the isotope peaks cannot be resolved) what is observed is the average mass.
- At high resolution where the isotopic peaks are fully resolved, then we can determine the monoisotopic mass for each one.

Take home question (due Jan 31st)

1. What is the monoisotopic mass of human hemoglobin alpha chain?
   - Hint: workout the empirical formula of hMyoglobin - its sequence can be obtained from record P69905 at http://www.ExPasy.org

2. What is the molecular weight of the most abundant species of human hemoglobin alpha?
   - Hint: assume that the abundance of $^{13}$C is 1.00% of total carbon atoms - do not worry about $^2$H or other isotopes.
Amino acid residues masses

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Mass</th>
<th>Amino Acid</th>
<th>Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>71.037</td>
<td>Leucine</td>
<td>113.084</td>
</tr>
<tr>
<td>Arginine</td>
<td>156.101</td>
<td>Lysine</td>
<td>128.094</td>
</tr>
<tr>
<td>Asparagine</td>
<td>114.043</td>
<td>Methionine</td>
<td>131.040</td>
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<tr>
<td>Aspartic acid</td>
<td>115.027</td>
<td>Phenylalanine</td>
<td>147.068</td>
</tr>
<tr>
<td>Cysteine</td>
<td>103.009</td>
<td>Proline</td>
<td>97.053</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>129.043</td>
<td>Serine</td>
<td>87.032</td>
</tr>
<tr>
<td>Glutamine</td>
<td>128.058</td>
<td>Threonine</td>
<td>101.048</td>
</tr>
<tr>
<td>Glycine</td>
<td>57.021</td>
<td>Tryptophan</td>
<td>186.079</td>
</tr>
<tr>
<td>Histidine</td>
<td>137.059</td>
<td>Tyrosine</td>
<td>163.063</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>113.084</td>
<td>Valine</td>
<td>99.068</td>
</tr>
</tbody>
</table>

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Blurring of protein space

- Identification using MALDI-TOF with MASCOT depends on:
  - Number of peptides recognized as being part of the protein
  - The mass accuracy of the peptides that are recognized
  - Pre-2000, an accuracy of better than 0.05 Da in a 1000 Da peptide (i.e., 50 ppm) was sufficient to distinguish the unknown protein from the other proteins in the databases at that time
  - Now, the protein information space has become more dense and MALDI-TOF is no longer adequate
  - Previously identified proteins may not be correct
ESI-MS and purity of peptides

Guarantees of purity based on observation of “a single peak by reverse-phase HPLC” and by “it gave the correct sequence when analyzed by Edman degradation” are hollow. The lower spectrum was of a “pure” HPLC peak. The method of purification was amended and the upper spectrum was obtained.

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Ionizing proteins and peptides

- \( ^+H_3NCHR_1CO(NHCHR_2CO)nNHCHR_2COOH \) is the ion that’s found in dilute acid solution
- If there are internal basic residues, then the ions will be of the form [M+nH]^{n+}, where n = 1, 2, etc.
- A tryptic peptide will have a N-terminal amino group and an amino group from Arg or Lys
  - If the peptide has a mol. wt. of 1000 Da, then the singly charged ion will have a \( m/z \) of 1001, whereas the doubly charged ion has a \( m/z \) of 501

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### ESI mass spectrum of ribonuclease

Cumulative MW estimate = 13,680.29
SD = 2.94

<table>
<thead>
<tr>
<th>Peak (m/z)</th>
<th>Intensity</th>
<th>Charge (est.)</th>
<th>Mol. Wt. (Est.)</th>
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<td>978.00</td>
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<td>1,053.00</td>
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<td>1,141.00</td>
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</table>

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### Calculation of molecular weights and ion states

- For two ions in a series for a peptide of molecular weight M, the lower m/z value (x) will be for the n+1 ion state and the larger m/z value (y) will be for the n+ ion state.
  - (1) \( \frac{M+n}{n} = y \)
  - (2) \( \frac{M+n+1}{n+1} = x \)

- Hence
  - (3) \( M+n = ny \) and \( M = ny-n \)
  - (4) \( M+n+1 = (n+1)x \) and \( M = (n+1)x-(n+1) \)

- Hence
  - \( ny-n = (n+1)x - (n+1) \)
  - \( ny-n-xn+n = x-1 \)
  - \( n(y-x) = x-1 \)
  - \( n = \frac{(x-1)}{(y-x)} \)

- The value of n can then be substituted in equation (1) to obtain the molecular weight of the peptide.

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**Deconvolution of MS data**

- When several proteins are present, then their multiply charged ion clusters overlap.
- Can this be overcome? - yes, use the MaxEntropy program provided by Micromass.

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**Oxidized Aprotinin ESI mass spectrum**

**Control Aprotinin ESI mass spectrum**

**Deconvoluted mass spectra**

Junlong Shao
Deconvolution of oxidized forms of β-lactoglobulin

Each ion is $[M+nH]^{n+}$

For 50+ charge state of a 50 kDa protein,

$$m/z = \frac{[50,000 + 50]}{50} = 1,001$$
**MaxEnt deconvolution of MWs**

![Graph showing mass spectra](image)

**Cytochrome C Modified by HNE**

**MALDI-TOF Mass Spectrum**

MALDI spectra usually contain only the molecular ion \([M+H]^-\). This is an advantage since it maximizes the signal, but is a disadvantage in that it gives no clues as to structure.
Summary of determining MW by ESI

- The multiple charge states of a protein allow:
  - Mol Wt of large proteins to be estimated
  - accurate estimation of mol wt (super SDS-PAGE gel)
- Important to remember that the protein sample must be free of salt
  - Typically, a sample is cleaned up on a short reverse-phase column prior to electrospray
  - Alternative, use ammonium acetate as buffer
Use of FT-MS in ESI of proteins

- The very high resolving power of FT-MS enables a direct measure of charge state of an individual ion since each peptide or polypeptide will have several/many isotope peaks.
- The distance in Da between successive isotope peaks of a multiply charged ion is the reciprocal of the number of charges.
**LC-MS of peptide mixtures**

- Analytical reverse phase column
  - 75 μm i.d. x 15 cm
- Flow rate 200 nl/min
- Acetonitrile gradient

**MUDPIT - Multi-Dimensional Protein Identification Technology**

- Hydrolyze everything!
- For a cell expressing 5,000 proteins, this leads to >100,000 peptides
- Can be fractionated, but still 10,000-20,000 to differentiate
- Enormous bioinformatics problem

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John Yates
Connecting CE and LC to MALDI analysis

- Creates 20 mm wide tracks that can be scanned by MALDI laser for MS analysis
- Parallel capture of effluents of 8 nanoLC separations on Mylar - can be scanned simultaneously by fast laser

Pros/Cons of laying down LC or EC separations on matrix plate

- Allows off-line analysis both in real time and then in a retrospective mode
- MALDI-TOF analysis is very fast
- Can do TOF-TOF MS-MS analysis
- BUT what happens chemically on the acidic environment on the surface of the plate during storage?
- Also, can the laser beam cause chemical changes?
Sequencing of peptides

- Using tandem mass spectrometry in a triple quadrupole, Q-tof, or ion trap instrument, the parent ion is first selected in the first quadrupole.
- The parent ion is collided with argon gas and it breaks into fragments (daughter ions).
- By identifying the daughter ions, the peptide amino acid sequence is inferred.

Tandem mass spectrometry on a triple quadrupole instrument

- Daughter ion spectra
- Parent ion spectra
- Multiple reaction ion scanning
**Triple quad versus Q-tof and sensitivity**

The quadrupole analyzer (Q3) is slow and insensitive - it's a filter - thus throws away large amounts of data.

The TOF detector collects all ions generated and yields fmol rather than pmol sensitivity.

Also gives far greater mass accuracy - from 1000 ppm on the triple quad to <20 ppm on the Q-tof.

Crucially important for automated interpretation of MS-MS spectra to yield amino acid sequence.

**Fragmenting a peptide**

\[
\text{NH}_3^+ \cdot \text{CHR}^1 \cdot \text{CO} \cdot \text{NH} \cdot \text{CHR}^2 \cdot \text{CO} \cdot \text{NH} \cdot \text{CHR}^3 \cdot \text{CO} \cdot \text{NH} \cdot \text{CHR}^4 \cdot \text{COOH}
\]

\[
\begin{align*}
\text{R}^1 \cdot \text{O} & \quad \text{R}^2 \\
\text{H}_2\text{N} - \text{C} - \text{C} - \text{N}^+ = \text{C}_x^2 \\
& \quad \text{H} \quad \text{H} \\
& \quad \text{H} \\
& \quad \text{R}^2 \cdot \text{O} \\
\text{H}_2\text{N} - \text{C} - \text{C} - \text{N}^+ = \text{C}^+ \\
& \quad \text{H} \quad \text{H} \\
& \quad \text{H} \\
\text{R}^1 \cdot \text{O} & \quad \text{R}^4 \\
\text{H}_2\text{N}^+ \cdot \text{C} - \text{C} - \text{N}^- \cdot \text{C} - \text{COOH} \\
& \quad \text{H} \quad \text{H} \\
& \quad \text{H} \\
\text{R}^2 \cdot \text{O} & \quad \text{R}^3 \\
\text{H}_2\text{N} - \text{C} - \text{C} - \text{N} = \text{C} = \text{O}^+ \\
& \quad \text{H} \quad \text{H} \\
& \quad \text{H} \\
\text{R}^1 \cdot \text{O} & \quad \text{R}^2^+ \\
\text{H}_2\text{N} - \text{C} - \text{C} - \text{N} = \text{C} - \text{NH}_3^+ \\
& \quad \text{H} \quad \text{H} \\
& \quad \text{H} \\
\text{R}^1 \cdot \text{O} & \quad \text{R}^4^+ \\
\text{H}_2\text{N}^+ \cdot \text{C} - \text{C} - \text{N}^- \cdot \text{C} - \text{COOH} \\
& \quad \text{H} \quad \text{H} \\
& \quad \text{H} \\
\text{R}^2 \cdot \text{O} & \quad \text{R}^3^+ \\
\text{H}_2\text{N} - \text{C} - \text{C} - \text{N} = \text{C} - \text{COOH} \\
& \quad \text{H} \quad \text{H} \\
& \quad \text{H} \\
\end{align*}
\]

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http://www.matrixscience.com/help/fragmentation_help.html
Amino acid residues masses

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<thead>
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<th>Amino Acid</th>
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<tbody>
<tr>
<td>Alanine</td>
<td>71.037</td>
<td>Leucine</td>
<td>113.084</td>
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<tr>
<td>Arginine</td>
<td>156.101</td>
<td>Lysine</td>
<td>128.094</td>
</tr>
<tr>
<td>Asparagine</td>
<td>114.043</td>
<td>Methionine</td>
<td>131.040</td>
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<tr>
<td>Aspartic acid</td>
<td>115.027</td>
<td>Phenylalanine</td>
<td>147.068</td>
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<td>Cysteine</td>
<td>103.009</td>
<td>Proline</td>
<td>97.053</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>129.043</td>
<td>Serine</td>
<td>87.032</td>
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<tr>
<td>Glutamine</td>
<td>128.058</td>
<td>Threonine</td>
<td>101.048</td>
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<tr>
<td>Glycine</td>
<td>57.021</td>
<td>Tryptophan</td>
<td>186.079</td>
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<tr>
<td>Histidine</td>
<td>137.059</td>
<td>Tyrosine</td>
<td>163.063</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>113.084</td>
<td>Valine</td>
<td>99.068</td>
</tr>
</tbody>
</table>

Identification of daughter ions and peptide sequence

N F L A G E K D N V R
b ions  262  375  446  503  632  760  875  989 1088 1187 1343
y ions  1361 1247 1100 987 916 859 730 602 487 373 274 175

Amino Acid Residues Masses

S. Barnes-UAB 1/24/06
Sequencing O-GlcNAc peptides by ECD FT-ICR-MS

Casein kinase II - AGGSTPVSSANMSG

Homework - write down the masses of the b and y ions

b_n = [residue masses + 1]  
y_n = [residue masses + H_2O + 1]