Lecture 8 (9/25/20)

- Reading: Ch3; 97-102
- Problems: Ch3 (text); 18, 19, 20, 23
  Ch3 (Study guide); **9**
  Ch4 (Study guide); **3**

NEXT

- Reading: Ch4; 119-122, 125-126, 131-133
  Ch4; 123-124, 130-131, 133, 137-138
- Problems: Ch4 (text); 2, 3, 4, 8, 13, 14

Lecture 8 (9/25/20)

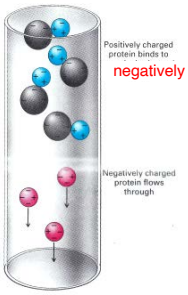
OUTLINE

I. Protein Structure
   A. Primary
      1. Determination
         a. Sequence determination; CHEMICAL
            i. Cleavage of peptides bonds
            ii. Amino acid composition and stoichiometry
            iii. Disrupt and determine number of chains;
            iv. Divide & Conquer;
            v. Edman Degradation
         b. Sequence determination; PHYSICAL
            i. Mass Spectrometry for proteins
            ii. Use of tandem MS/MS for sequence determination
            iii. Isolation of proteins by 2D PAGE; Isoelectric focusing x SDS-PAGE
         c. Sequence determination; BIOLOGICAL
            i. Genome sequenced
            ii. Bioinformatics to predict protein sequences in predicted genes
            iii. Use of CHEMICAL and/or PHYSICAL methods to get partial sequence

Determination of primary structure

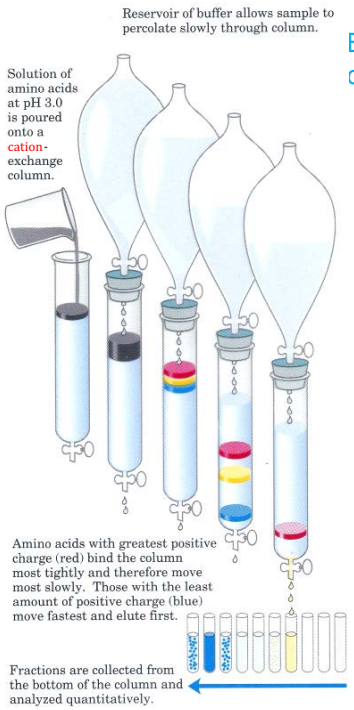
1) Purify protein
2) Determine the amino-acid composition, including stoichiometry
3) Disrupt structure (2°, 3°, 4°, and disulfides)
4) Determine the number of peptide chains by counting number of amino terminal ends
5) Divide into fragments and determine sequence
6) Divide into different set of fragments and determine sequence
7) Determine overlaps and piece original sequence back together

Ion Exchange Chromatography: Example of amino-acid mixture



If this were a mixture of G, K, and D, what would the order of elution be?

1st= D, 2nd = G, 3rd = K



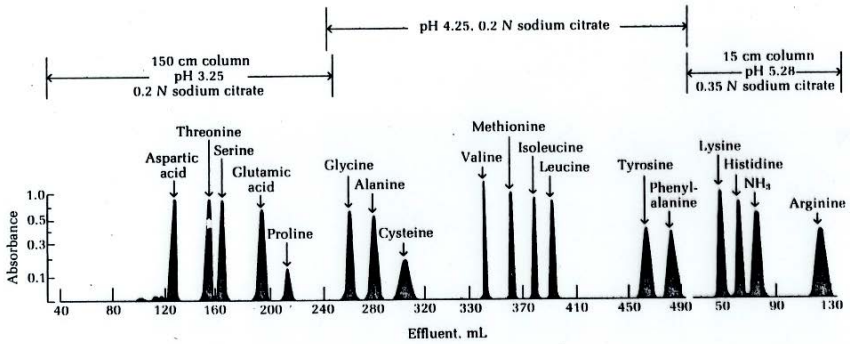
Basis = charge

Determination of primary structure

Wm H. Stein
Stanford Moore

Amino Acid Composition

Figure 5-15
Automatically recorded chromatographic analysis of amino acids on a cation-exchange resin. The elution is carried out with different buffers of successively higher pH. The effluent is caught in small volumes, and the amino acid content of each tube is automatically analyzed. The area under each peak is proportional to the amount of each amino acid in the mixture.



Absorbance is at 570 nm (purple). How did they make the amino acid residues colored? .....ninhydrin

How many AA are "resolved?" 18

Where are the other 2? .....Asn & Gln have already been converted to Asp & Glu, respectively

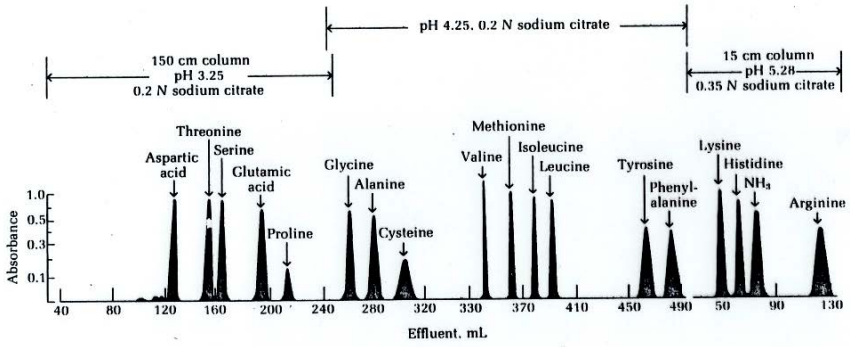
Determination of primary structure

Wm H. Stein
Stanford Moore

Amino Acid Composition

Figure 5-15
Automatically recorded chromatographic analysis of amino acids on a cation-exchange resin. The elution is carried out with different buffers of successively higher pH. The effluent is caught in small volumes, and the amino acid content of each tube is automatically analyzed. The area under each peak is proportional to the amount of each amino acid in the mixture.

Cation exchanger=Dowex-50 (sulfonic acid on polystyrene)



Calculate the "mole%" or "mole fraction"; in other words the stoichiometry:

Example; protein MW = 11,000 Da

Approximate #AA = 11,000/110 (Ave MW for AA) = 100 AA

If 10% of A_{570} is 10% for Arg: 100 x 0.10 = 10 Arg in protein

Determination of primary structure

1) Purify protein
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7) Determine overlaps and piece original sequence back together

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Determination of primary structure

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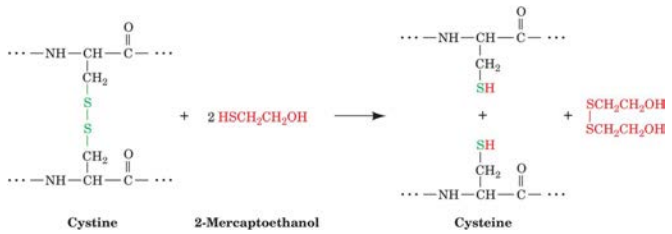
Disrupt structure (2°, 3°, 4°, and disulfides)

What holds these levels of structure together? .....non-covalent bonds (H-bonds, van der Waals, ionic, hydrophobic)

What have you used in the lab that might disrupt non-covalent bonds? .....Urea, SDS, pH extremes, heat, etc.

What about the covalent S-S bond? .....2-mercaptoethanol (β-mercaptoethanol, BME) or dithothreitol (DTT).



To keep disulfides from reforming…..
1) keep BME at high concentration in buffers
2) alkylate the SH groups

Determination of primary structure

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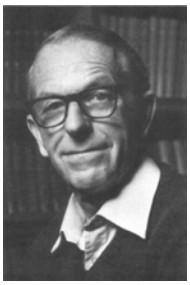
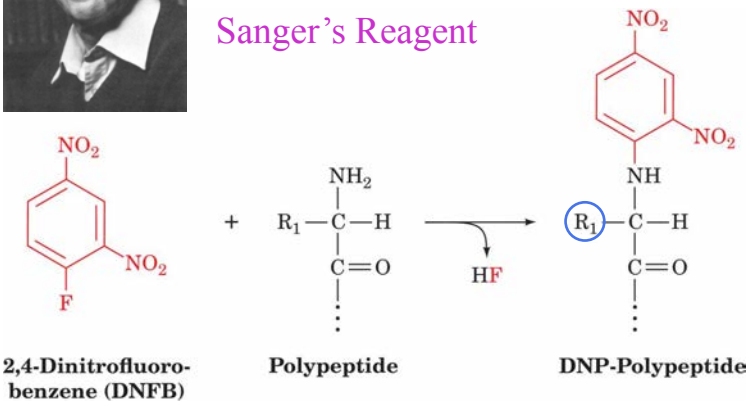
Determine the number of peptide chains by counting number of amino terminal ends



Frederick Sanger
(1918-2013)

Example:
small tripeptide
- AA comp = A,K,L
- C-term = K
- DNP-A
- Sequence is A-L-K

Sanger's Reagent

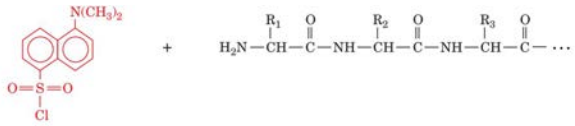


2,4-Dinitrofluoro-benzene (DNFB) **Polypeptide** **DNP-Polypeptide**

Absorbs at 353 nm (yellow)

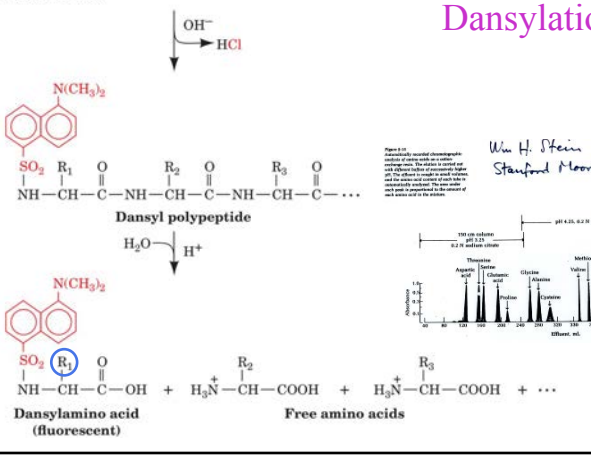
Fig 3-26

Determine the number of peptide chains by counting number of amino terminal ends



5-Dimethylamino-1-naphthalenesulfonyl chloride (dansyl chloride) **Polypeptide**

Dansylation



Excitation at 330 nm
Emission at 519 nm

Determination of primary structure

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Determination of primary structure

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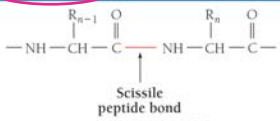
Divide into fragments:

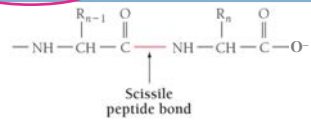
Proteolytic Cleavage

TABLE 5-4 Specificities of Various Endopeptidases



Enzyme	Source	Specificity	Comments
Trypsin	Bovine pancreas	R_{n-1} = positively charged residues: Arg, Lys; $R_n \neq$ Pro	Highly specific
Chymotrypsin	Bovine pancreas	R_{n-1} = bulky hydrophobic residues: Phe, Trp, Tyr; $R_n \neq$ Pro	Cleaves more slowly for R_{n-1} = Asn, His, Met, Leu
Elastase	Bovine pancreas	R_{n-1} = small neutral residues: Ala, Gly Ser, Val; $R_n \neq$ Pro	
Thermolysin	*Bacillus thermoproteolyticus*	R_n = Ile, Met, Phe, Trp, Tyr, Val; $R_{n-1} \neq$ Pro	Occasionally cleaves at R_n = Ala, Asp, His, Thr; heat stable
Pepsin	Bovine gastric mucosa	R_n = Leu, Phe, Trp, Tyr; $R_{n-1} \neq$ Pro	Also others; quite nonspecific; pH optimum = 2
Endopeptidase V8	*Staphylococcus aureus*	R_{n-1} = Glu	

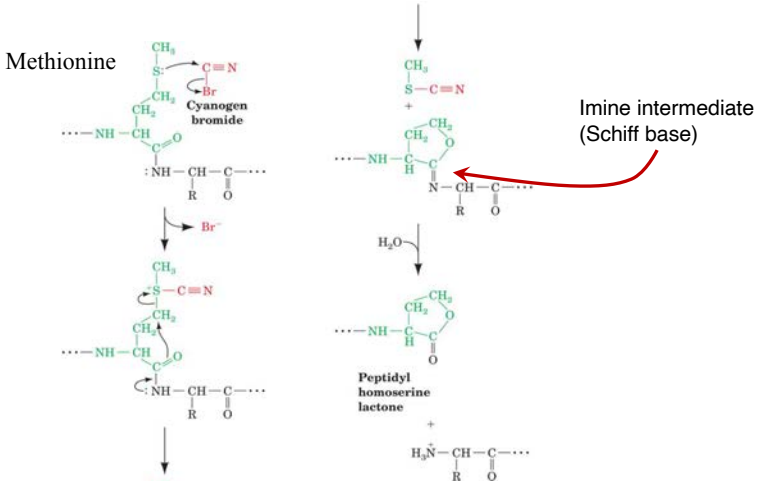
"TABLE 5-5" Specificities of Exopeptidase



Carboxylpeptidase A	Bovine pancreas	R_n = C-terminal; $R_{n-1} \neq$ Pro

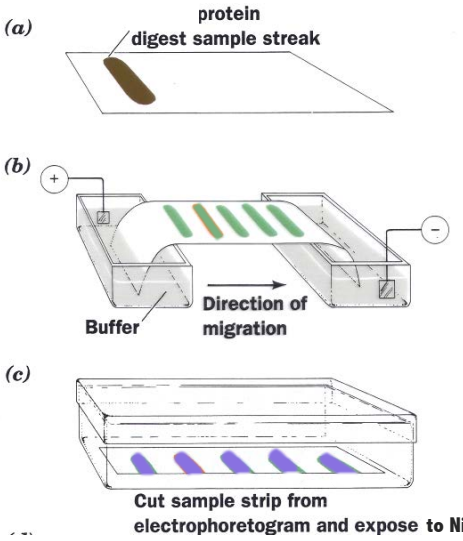
Divide into fragments:

Cyanogen Bromide Cleavage



Similar result as an endopeptidase, but leaves a **homoserine lactone** as the C-terminal residue

Separation and isolation of peptide fragments



Example: paper electrophoresis

Also, TLC, silica gel, etc.

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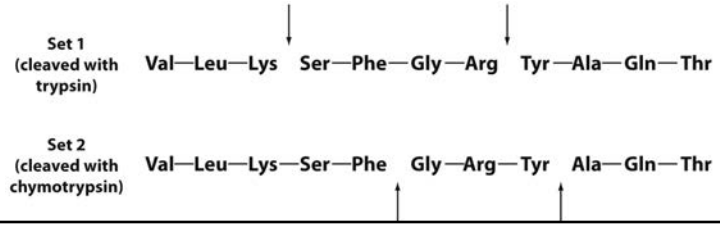
Determination of primary structure

- Determine amino-acid composition
- Dansyl chloride or FDNB to determine amino-termini and number
- Proteases: Cleaves peptide bonds only after specific residues.
- Cleave protein with 2 different proteases.
- Sequence fragments with Edman degradation. Piece together sequence from overlapping fragments.



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Determination of primary structure: Divide & Conquer

(Ala$_2$, Gly, Lys$_2$, Phe, Thr, Trp, Val)

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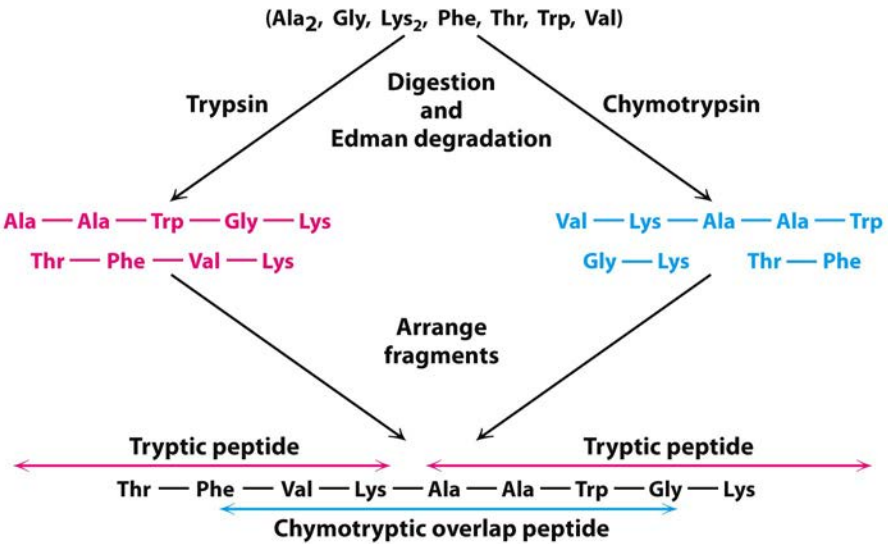
Determination of primary structure: Divide & Conquer



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Determination of primary structure

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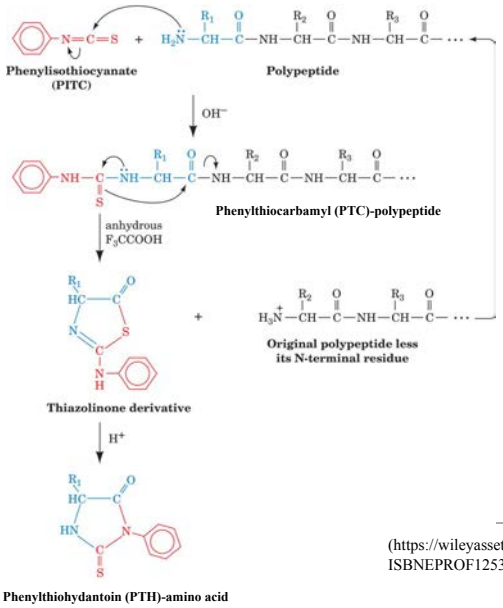
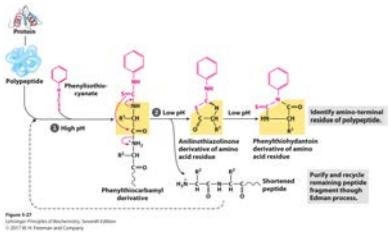
Determine the Sequence: Edman degradation





Edman Degradation Animated Figure

(https://wileyassets.s3.amazonaws.com/Voet_Fundamentals_of_Biochemistry_5e_ISBNEPROF12533/media/animated_figures/ch05/5-15.html)

 Figure 5-16 WILEY

Determination of primary structure

1) Purify protein
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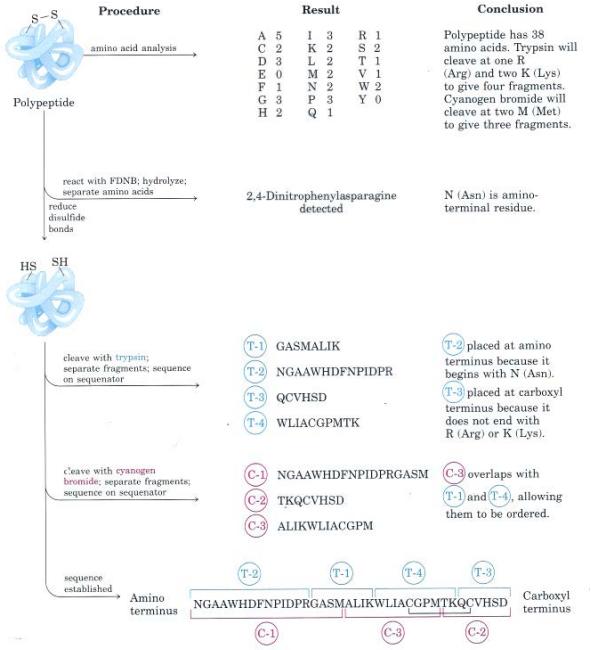


Determine overlaps and piece original sequence back together

Wiley Guided_explorations

Determination of primary structure

THREE basic ways to know the primary structure. Only the CHEMICAL method will give the entire covalent structure, including any disulfide bonds. But other methods are more sensitive. One can classify these methods by:

CHEMICAL
PHYSICAL
BIOINFOMATICAL

We just went through the CHEMICAL.
The PHYSICAL method still requires the same strategy, including purification, fragmentation, chromatography, and alignment.

But, instead of an Edman degradation the use of tandem Mass Spectrometry (MS) is employed.

Lets look at the use of MS in biochemistry

- Ions "fly" in a vacuum toward a target with a velocity $\propto$ z/m (charge-to-mass ratio)
- Molecules with higher charge and lower mass get detected first.
- Molecules with a lower charge and higher mass get detected last.
- Plotted as m/z to read peaks from left to right
- Instruments can distinguish molecules with same charge by < 1 Da

Determine the Sequence: Tandem MS

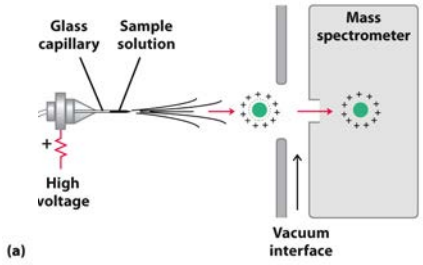
The major problem in using MS for macromolecules is getting them to "fly" in a vacuum with a charge.

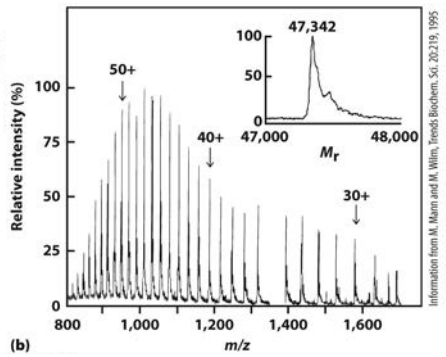
TWO major methods:

1) Electro-Spray Ionization (ESI)
2) Matrix-Assisted Laser-Desorption Ionization (MALDI)

What is MALDI?

ESI





Mass = (m/z) x z

$(m/z_A) \times z_A = (m/z_B) \times z_B$

$893.3z = 848.7(z+1)$

$893.3z = 848.7z+848.7$

$848.7 = z(893.3–848.7) = z44.6$

$848.7/44.6 = 19 = z$

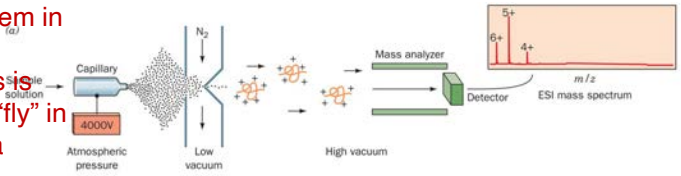
Mass = 893.3x19 = 16,973
Mass = 848.7x20 = 16,974
Mass = 1696.3x10 = 16,963

Determine the Sequence: Tandem MS

The major problem in using MS for macromolecules is getting them to "fly" in a vacuum with a charge.

TWO major methods:

1) Electro-Spray Ionization (ESI)
2) Matrix-Assisted Laser-Desorption Ionization (MALDI)



ESI

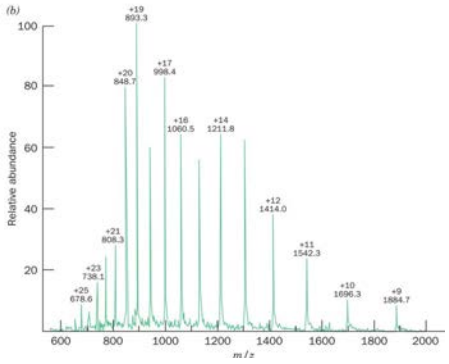


Figure 5-17

What is MALDI?

Mass = (m/z) x z

$(m/z_A) \times z_A = (m/z_B) \times z_B$

$893.3z = 848.7(z+1)$

$893.3z = 848.7z+848.7$

$848.7 = z(893.3–848.7) = z44.6$

$848.7/44.6=19 = z$

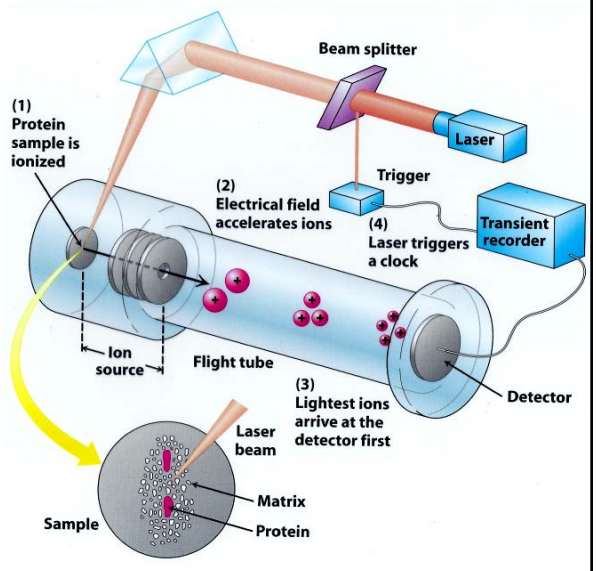
Mass = 893.3x19 = 16,973

Mass = 848.7x20 = 16,974

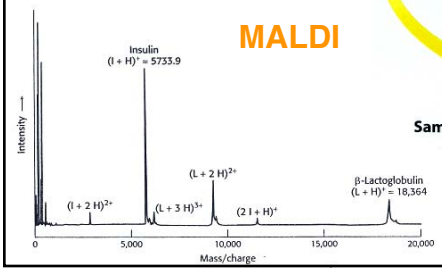
Mass = 1696.3x10 = 16,963

Determine the Sequence: Tandem MS

Matrix-Assisted Laser-Desorption Ionization (MALDI)

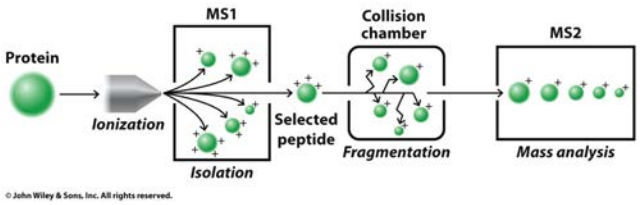


MALDI

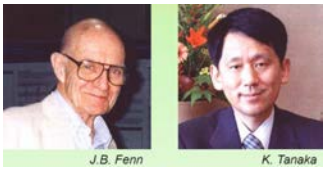


How do you use MS to get protein sequences?

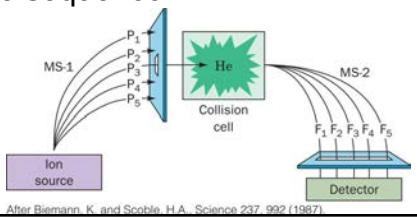
Determine the Sequence: Tandem MS



- Mass spectrometry uses mass-to-charge ratio of different ions to determine mass
- Tandem MS-MS: First selects a peptide, then fragmentation, and second determines mass of fragments
- By comparing all of fragments, those that different by mass of one amino acid to determine sequence



Nobel Prize in Chemistry 2002



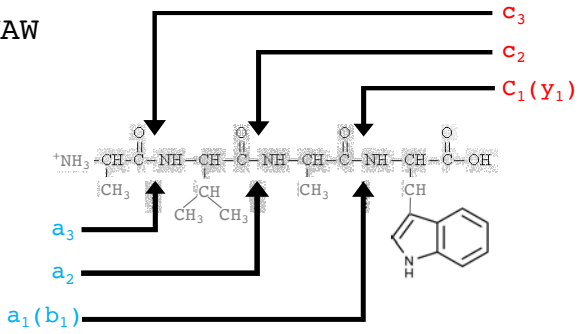
After Biemann, K. and Scoble, H.A., Science 237, 992 (1987)

Determine the Sequence: Tandem MS

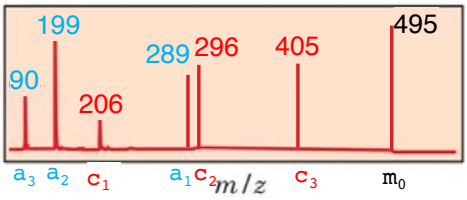
- EXAMPLE

AVAW

AA	MW
Ala	90
Val	109
Trp	206



AVAW



Determine the Sequence: Tandem MS

- EXAM

AA	MW
Ala	90
Val	109
Trp	206

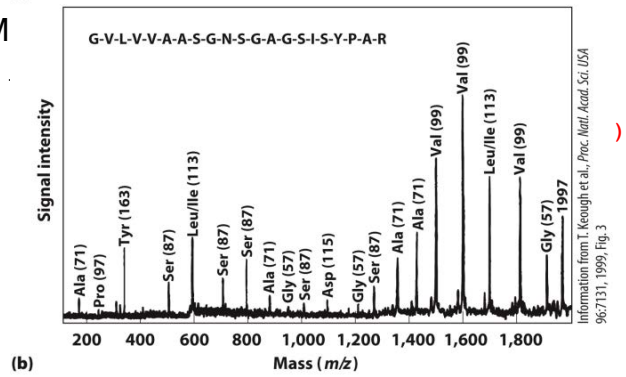
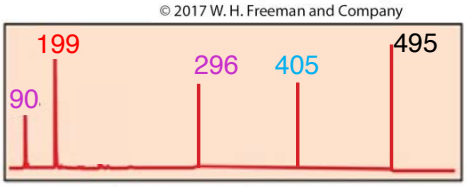
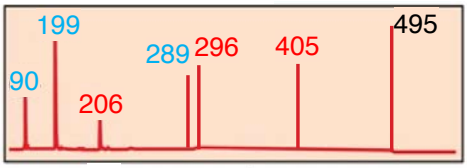


Figure 3-31
Lehninger Principles of Biochemistry, Seventh Edition
© 2017 W. H. Freeman and Company



a_3 c_2 a_2 m/z a_1 m_0
c_1 c_3



a_3 a_2 c_1 a_1 c_2 m/z c_3 m_0

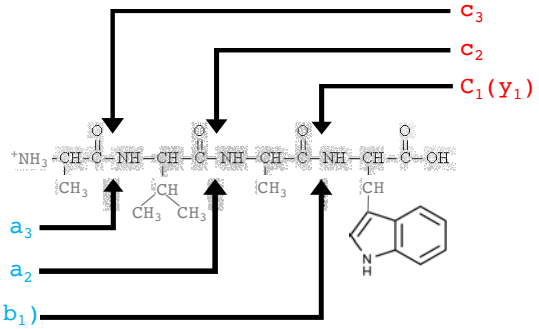
There is an issue with K & Q, which have the same MW!

Determine the Sequence: Tandem MS

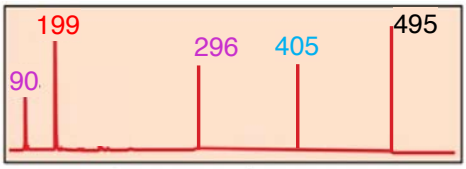
- EXAMPLE

AVAW

AA	MW
Ala	90
Val	109
Trp	206

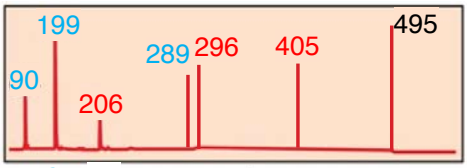


AWVA



a_3 c_2 a_2 m/z a_1 m_0
c_1 c_3

AVAW



a_3 a_2 c_1 a_1 c_2 m/z c_3 m_0

There is an issue with K & Q, which have the same MW!