

# Fundamentals of Protein Chemistry

Amino Acid and Peptide Chemistry

Transcription and Translation

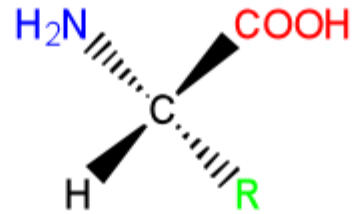
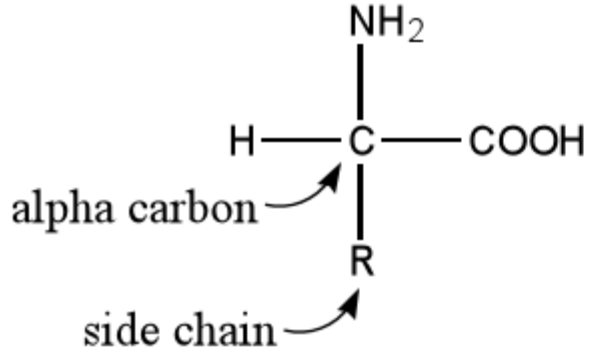
Post-Translational Modifications

Classical Analytical Methods

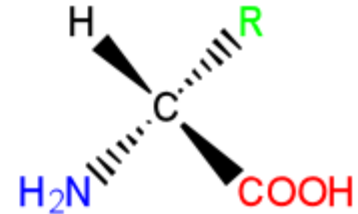
In-Gel Proteolysis

# Amino Acids are the Basic Structural Units of Proteins

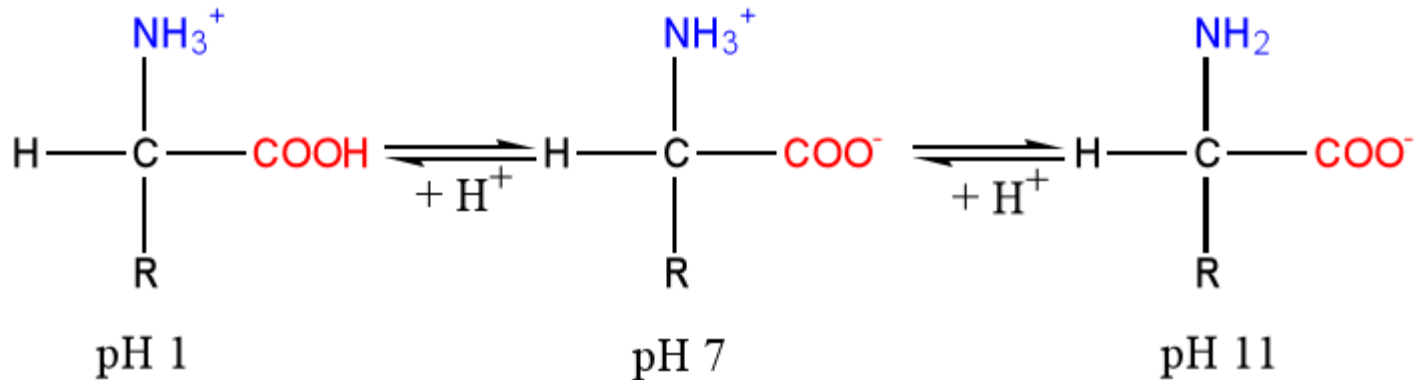
General Structure  
of an Amino Acid



L-Isomer



D-Isomer



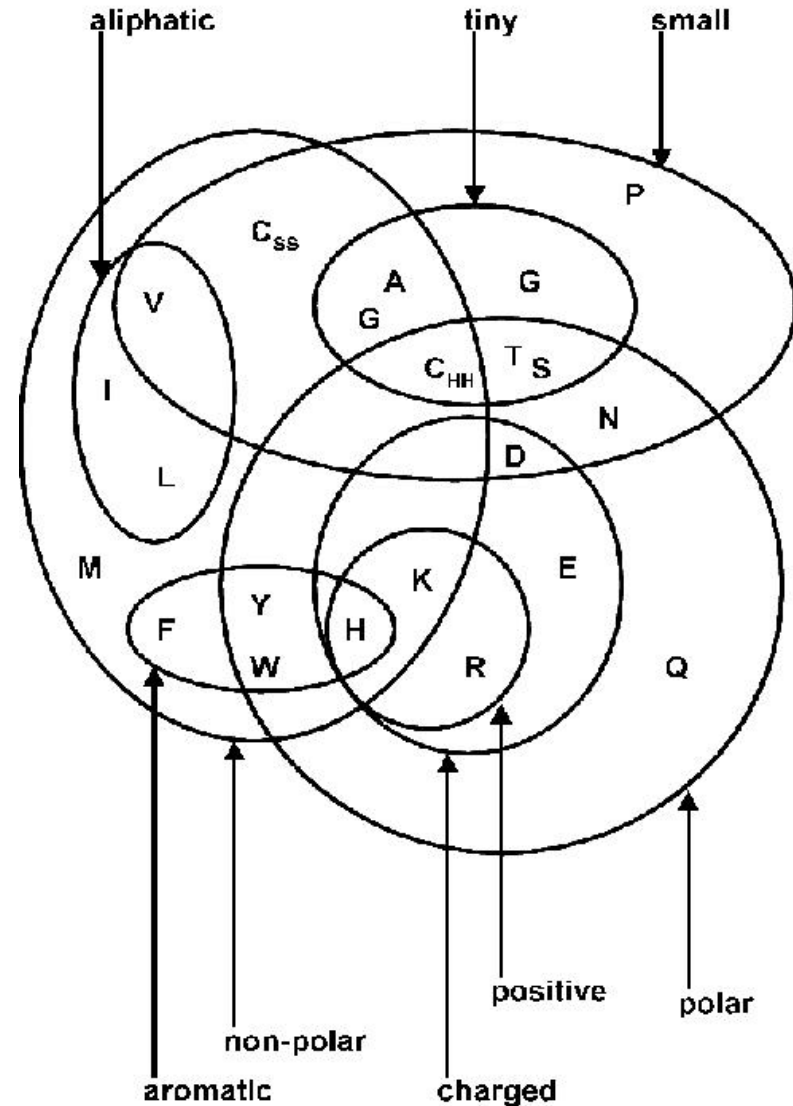
The Ionization State of Amino Acids Changes with pH

# Names, Abbreviations, and Properties of The Twenty Amino Acids

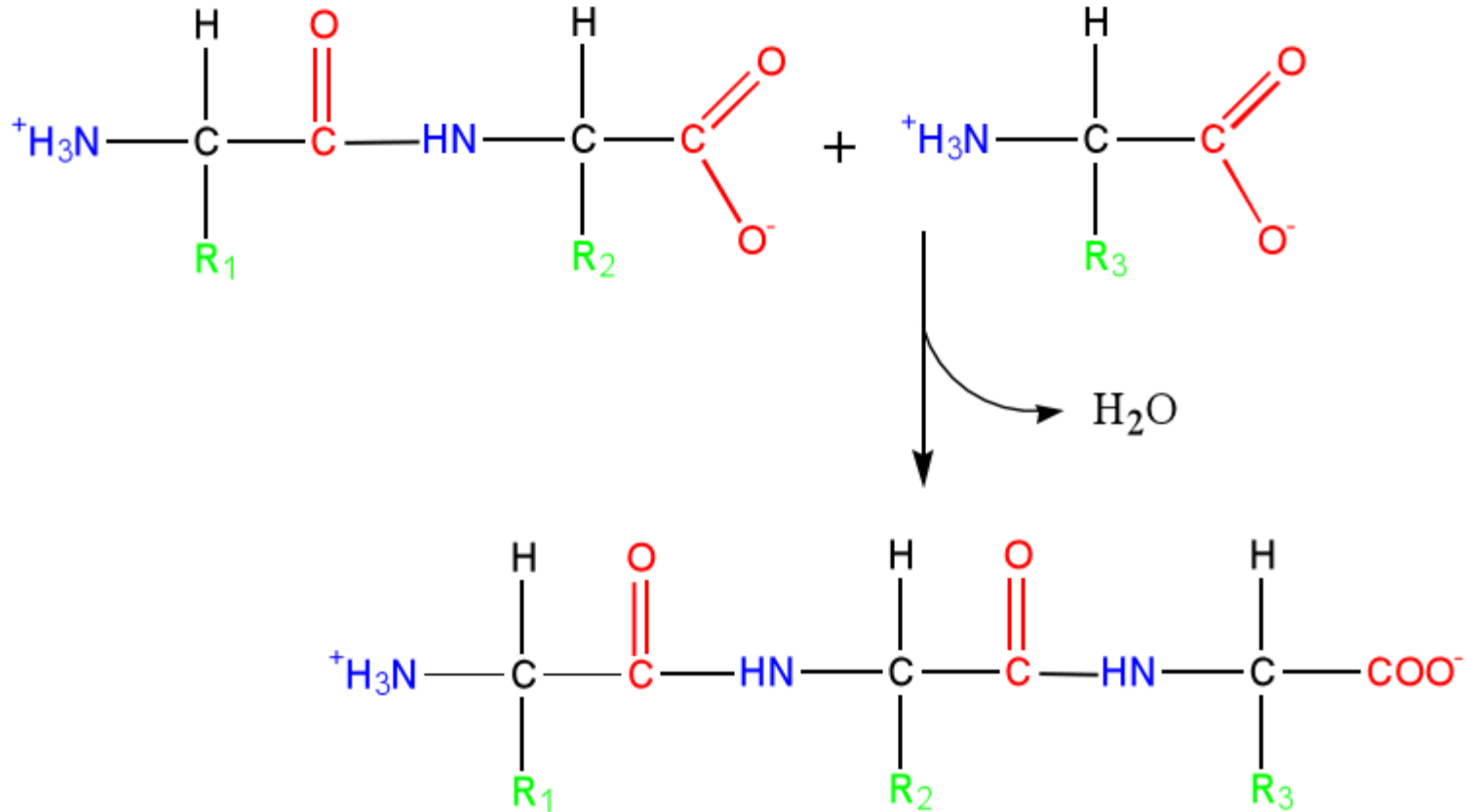
Name			Side Chain pKa	pI
Alanine	Ala	A		6.11
Arginine	Arg	R	12.5	10.76
Aspartic Acid	Asp	D	3.9	2.98
Asparagine	Asn	N		
Cysteine	Cys	C	8.3	5.02
Glutamic Acid	Glu	E	4.3	3.08
Glutamine	Gln	Q		
Glycine	Gly	G		6.06
Histidine	His	H	6.0	7.64
Isoleucine	Ile	I		6.04
Leucine	Leu	L		6.04
Lysine	Lys	K	10.8	9.47
Methionine	Met	M		5.74
Phenylalanine	Phe	F		5.91
Proline	Pro	P		6.3
Serine	Ser	S		5.68
Threonine	Thr	T		
Tryptophan	Trp	W		5.88
Tyrosine	Tyr	Y	10.9	5.63
Valine	Val	V		6.00

# The Twenty Amino Acid Side Chains Vary Significantly

The twenty naturally occurring amino acids that make up proteins can be grouped according to many criteria, including hydrophobicity, size, aromaticity, or charge.



# Amino Acids are Linked by Amide Bonds to Form Peptide Chains



General Structure of a Peptide

# Acid-Base Chemistry in Protein Characterization

The net charge of a peptide or protein at any pH depends on the combined pK values for its amino acids and terminal groups.

$$\text{pH} = \text{pK} + \log [\text{A}^-]/[\text{HA}]$$

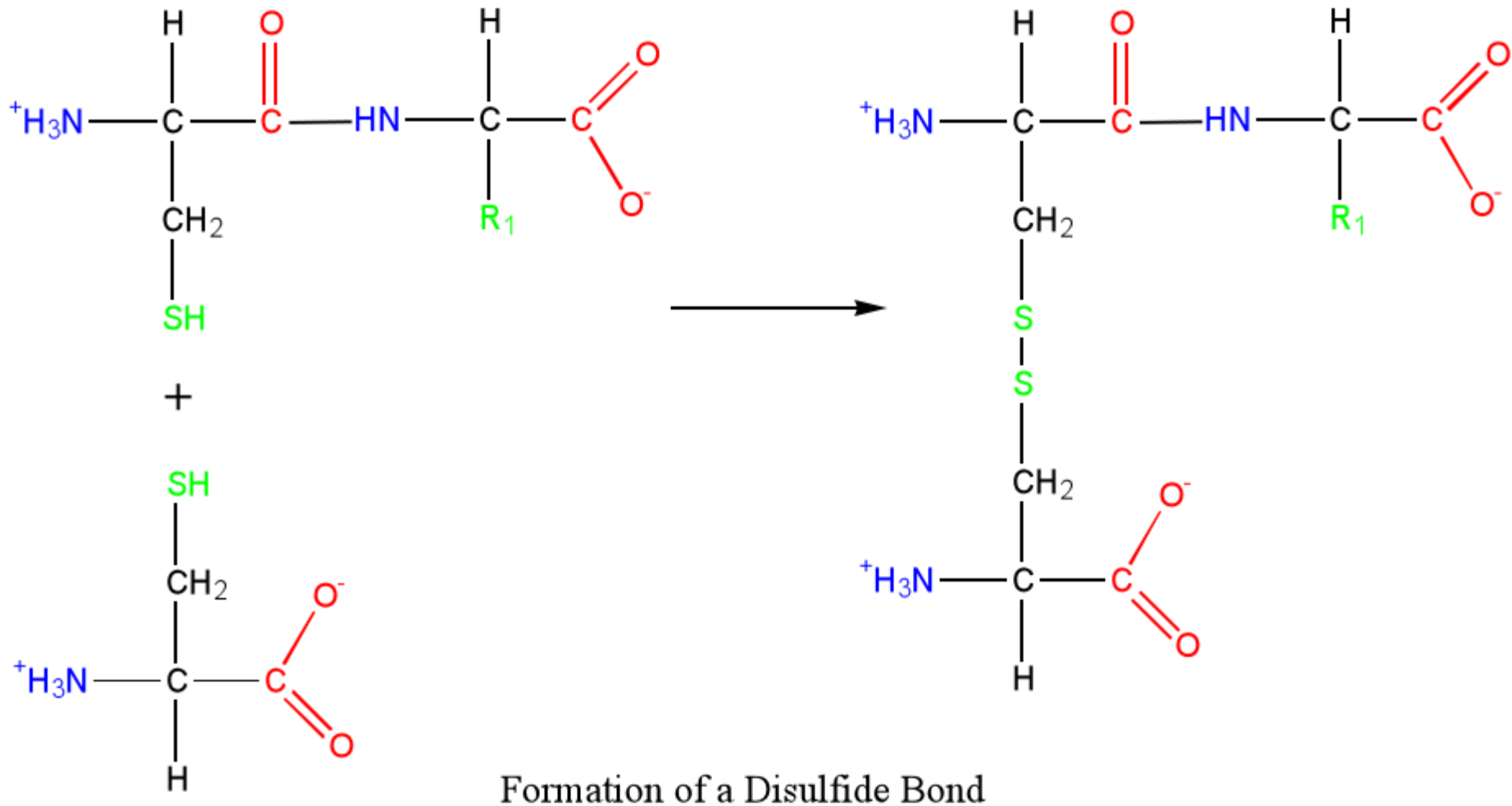
<b>pK of alpha-COOH groups:</b>	<b>1.8 - 2.4</b>
<b>pK of alpha-NH<sub>2</sub> groups:</b>	<b>9.0 - 10.8</b>
<b>pK of ionizable side chains:</b>	<b>3.9 - 12.5</b>

The isoelectric point is the pH at which there is no net charge.

It is important to remember how protein and peptide pK values affect chemistry and separations:

- Chemical Modification (e.g. Reduction / Alkylation)**
- Proteolysis (e.g. specificity of Glu-C)**
- Chromatography (e.g. Ion Exchange)**
- 2D Gel Separations (Isoelectric Focusing)**
- Ionization for Mass Spectrometry (e.g. MALDI-TOFMS)**

# Peptide Chains are Linked by Disulfide Bonds Between Cysteines

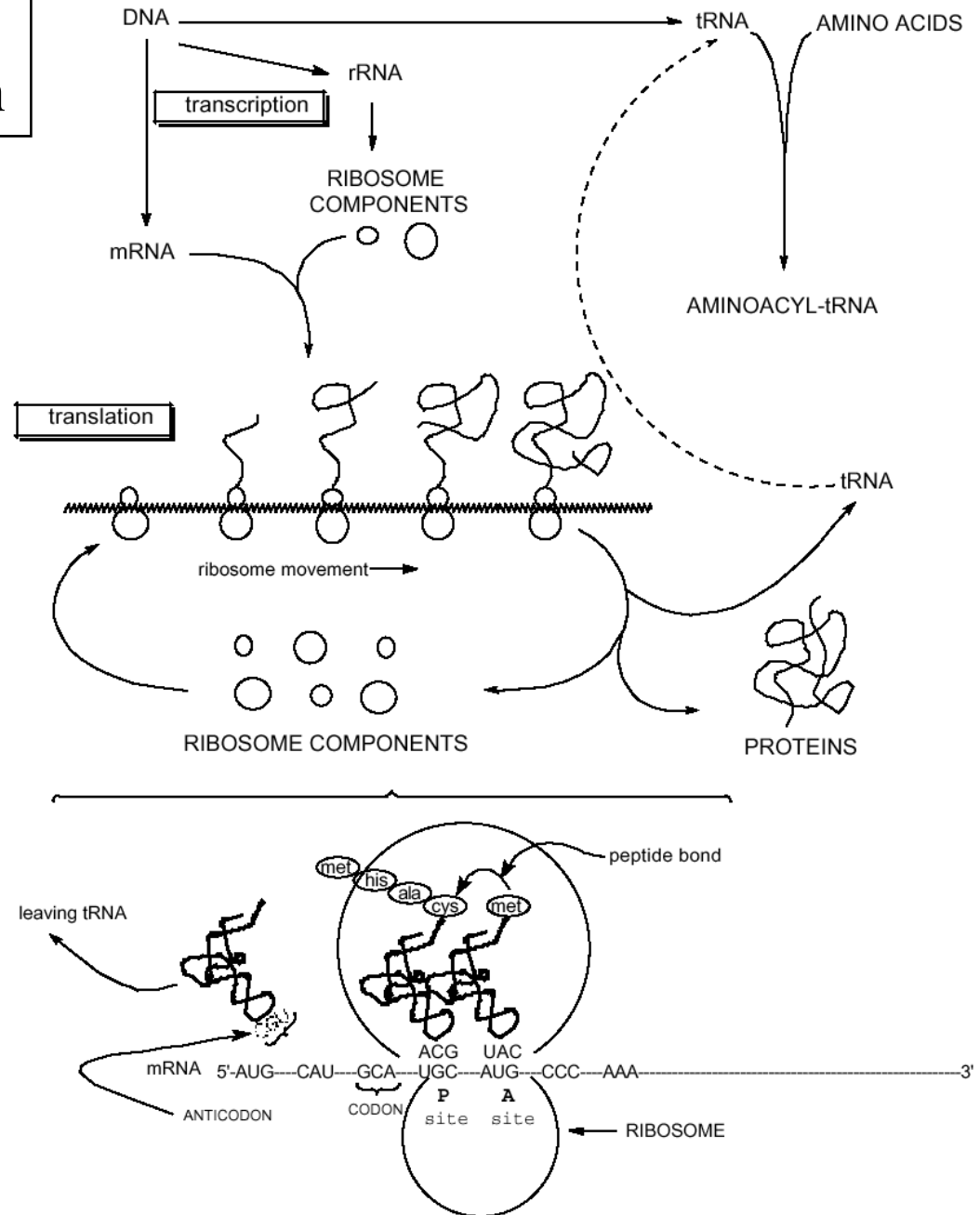


# Protein Synthesis: Transcription & Translation

Messenger RNA is synthesized in the nucleus and exits through a nuclear pore.

Ribosomes assemble along the mRNA and translate it into a peptide sequence.

The growing peptide may be directed into the lumen of the endoplasmic reticulum (ER) for processing.





# Translation: Amino Acid Codons

Phe  
TTT  
TTC

Leu  
TTA  
TTG  
CTT  
CTC  
CTA  
CTG

Ile  
ATT  
ATC  
ATA

Met  
ATG

Val  
GTT  
GTC  
GTA  
GTG

Ser  
TCT  
TCC  
TCA  
TCG  
AGT  
AGC

Pro  
CCT  
CCC  
CCA  
CCG

Thr  
ACT  
ACC  
ACA  
ACG

Ala  
GCT  
GCC  
GCA  
GCG

Tyr  
TAT  
TAC

His  
CAT  
CAC

Gln  
CAA  
CAG

Asn  
AAT  
AAC

Lys  
AAA  
AAG

Asp  
GAT  
GAC

Glu  
GAA  
GAG

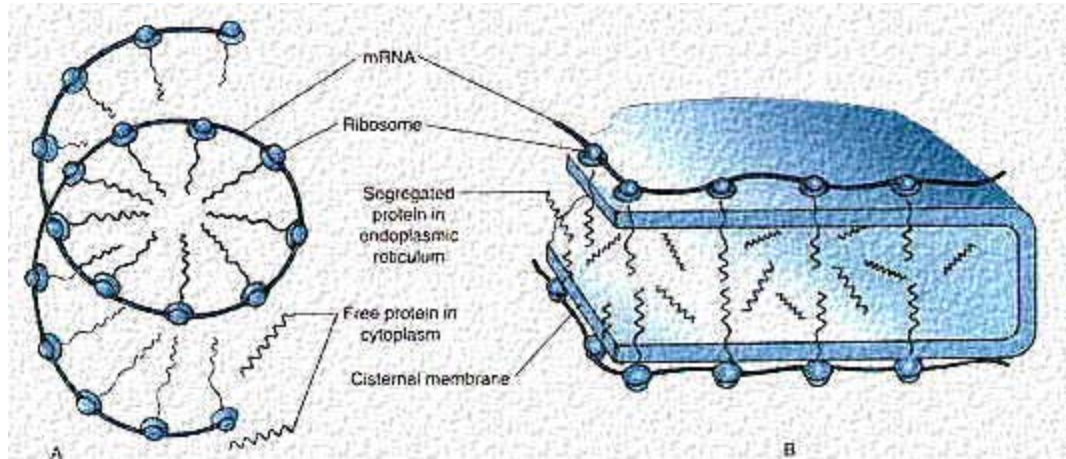
Cys  
TGT  
TGC

Trp  
TGG

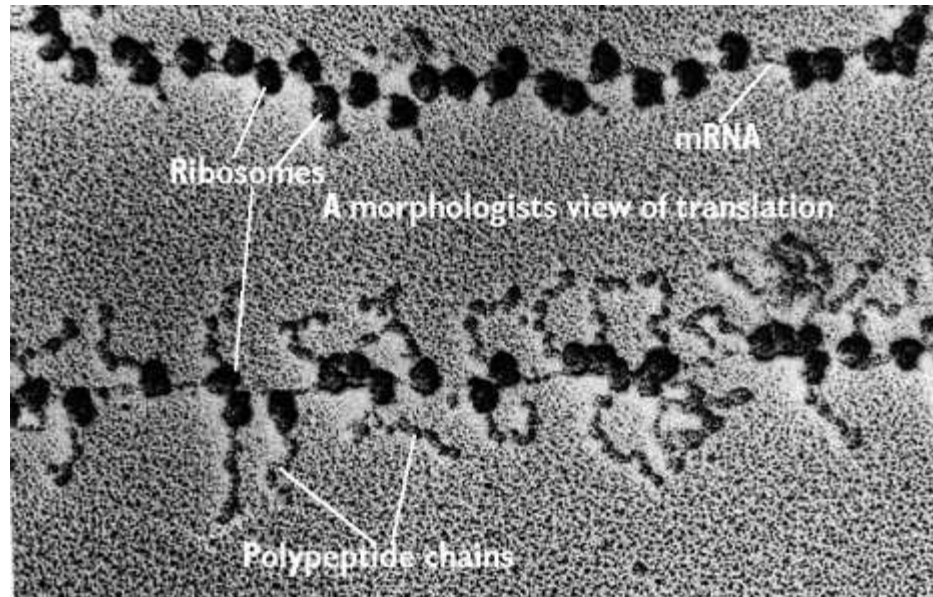
Arg  
CGT  
CGC  
CGA  
CGG  
AGA  
AGG

Gly  
GGT  
GGC  
GGA  
GGG

# Protein Synthesis: Translation



Histology by Bergman, R.A., Afifi A.K. and Heidger, P.M. Saunders Publishing, Philadelphia, Pa, 1995

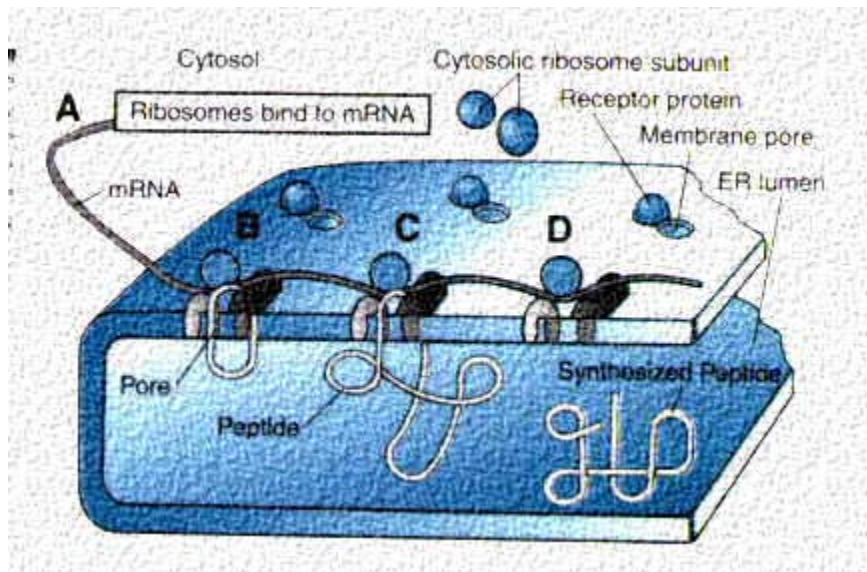


Molecular Biology of the Cell by Bruce Alberts, Dennis Bray, Julian Lewis, Martin Raff, Keith Roberts, and James D. Watson, Garland Publishing, NY 1994

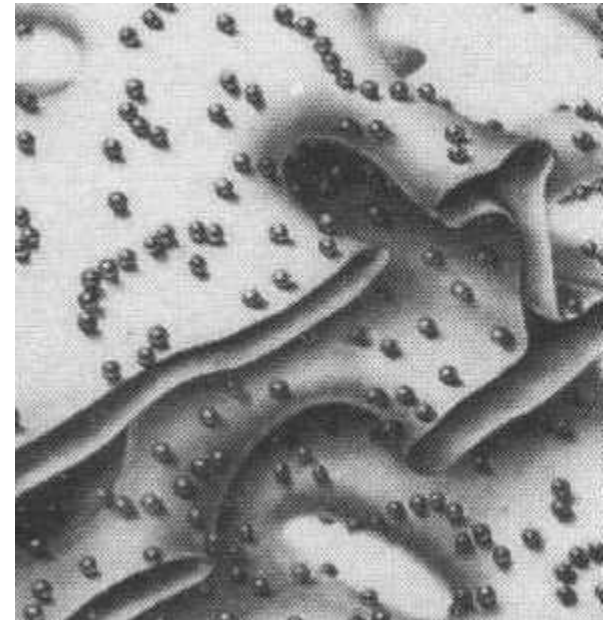
# Protein Synthesis: Translation

As a peptide grows into the ER, it is folded and modified.

The ER has multiple compartments in which specific binding proteins and enzymes act on the new protein molecule.



Histology by Bergman, R.A., Afifi A.K. and Heidger, P.M. Saunders Publishing, Philadelphia, Pa, 1995

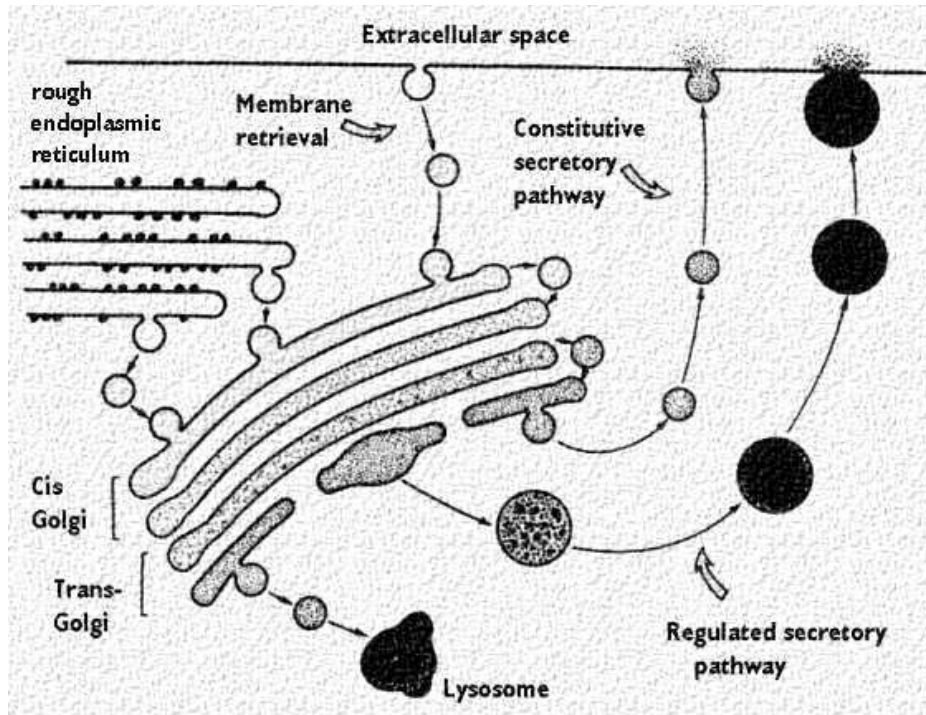


A Textbook of Histology by D Fawcett. Chapman and Hall, N.Y. 1994

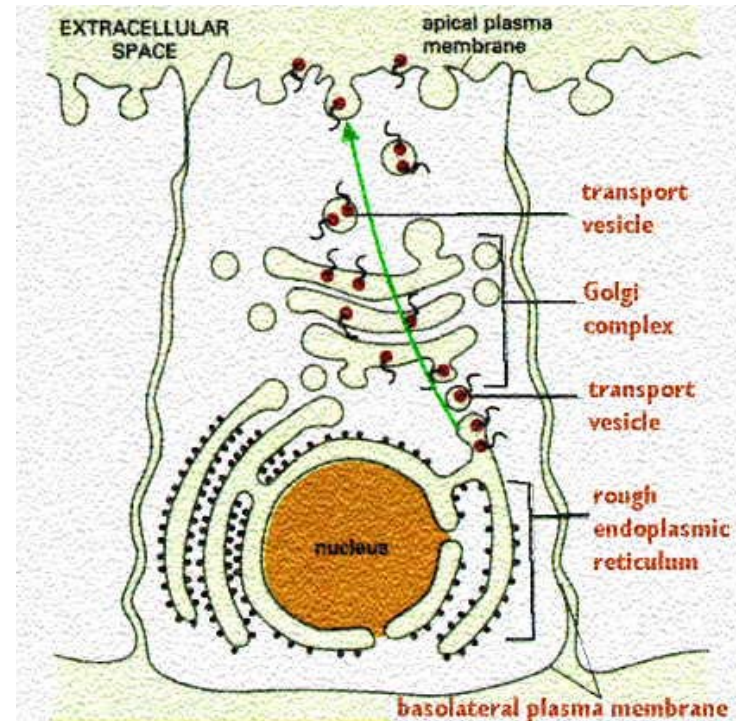


# Protein Folding, Modification, and Transport

A newly synthesized protein can be transported from the ER to the Golgi Apparatus, another complex series of compartments in which modifications are made. The Golgi is important for determining the disposition of proteins.



A Textbook of Histology by D Fawcett. Chapman and Hall, N.Y. 1994



Molecular Biology of the Cell by Bruce Alberts, Dennis Bray, Julian Lewis, Martin Raff, Keith Roberts, and James D. Watson, Garland Publishing, NY 1994

# Protein Post-Translational Modification

Protein modifications performed by “extra-translational” processes.

Cannot be definitively predicted from DNA sequence

Can involve very complex systems of enzymes

In some cases, “consensus” sites of modification can be identified

Ubiquitous in eukaryotes

Frequently critical for:

initiation or modulation of biological activity

transport, secretion

# Protein Post-Translational Modification

Proteolytic Cleavage **N-term Met of all proteins removed by aminopeptidases**

● N-terminal Acylation **formyl, acetyl, myristyl, etc. by acyltransferases**

● Glycosylation **Asn, Ser, and Thr**

Sulfation **Tyr**

● Phosphorylation **Ser, Thr, and Tyr**

Carboxyl Terminal Amidation

Hydroxylation **Pro, Lys, Asp**

N-Methylation **Lys, Arg, His, Gln**

Carboxylation **Glu, Asp**

● Modifications introduced by us **Met [O], Cys-acrylamide**

<http://abrf.org/ABRF/ResearchCommittees/deltamass/deltamass.html>

Exhaustive list maintained by Ken Mitchelhill

# Protein Mutations

Single nucleotide changes can result in amino acid substitutions

Single amino acid substitutions can result in alterations in:

- Protein 3D structure

- Molecular Weight

- Isoelectric Point

- Proteolysis by specific enzymes

- Molecular weights of proteolytic fragments

**Protein Prospector, UCSF MS Facility**

**<http://prospector.ucsf.edu>**

**Table of Mass Shifts due to Single AA mutations**

**<http://prospector.ucsf.edu/ucsfhtml3.4/misc/mutation.htm>**

# Robust Analytical Methods for Protein Characterization

## Amino Acid Analysis

Acid Hydrolysis followed by derivatization and HPLC

- Determines the precise molar ratios of amino acids present
- Can also be used to accurately determine concentration  
Asp/Asn and Glu/Gln are not distinguished  
Cysteine and Tryptophan are problematic in some methods

## Amino-Terminal Sequencing by Edman Degradation

- Very sensitive
- Standard method- still best approach to NH<sub>2</sub>-terminus  
Very steep learning curve to do it well  
Blocked proteins cannot be analyzed  
Mixtures are challenging  
Peptides with long repeats are problematic  
PTMs are often missed but can be dealt with  
Often not competitive with MS for internal sequence



# Robust Analytical Methods for Protein Characterization

## Polyacrylamide Gel Electrophoresis

- A crude measure of molecular weight and purity
- Analytical or preparative separations
- Coupled with Blotting- sensitive & selective detection

## Isoelectric Focusing

Analytical or preparative separations

Used for mapping disease markers (e.g. CGDs)

Variety of pH gradients

Automated, high throughput instruments

## Two Dimensional IEF – PAGE

- Orthogonal separations- large separation space
- Detection of small changes in complex samples
- Separation of post-translationally modified proteins
- Dynamic Range problems due to sample loading capacity

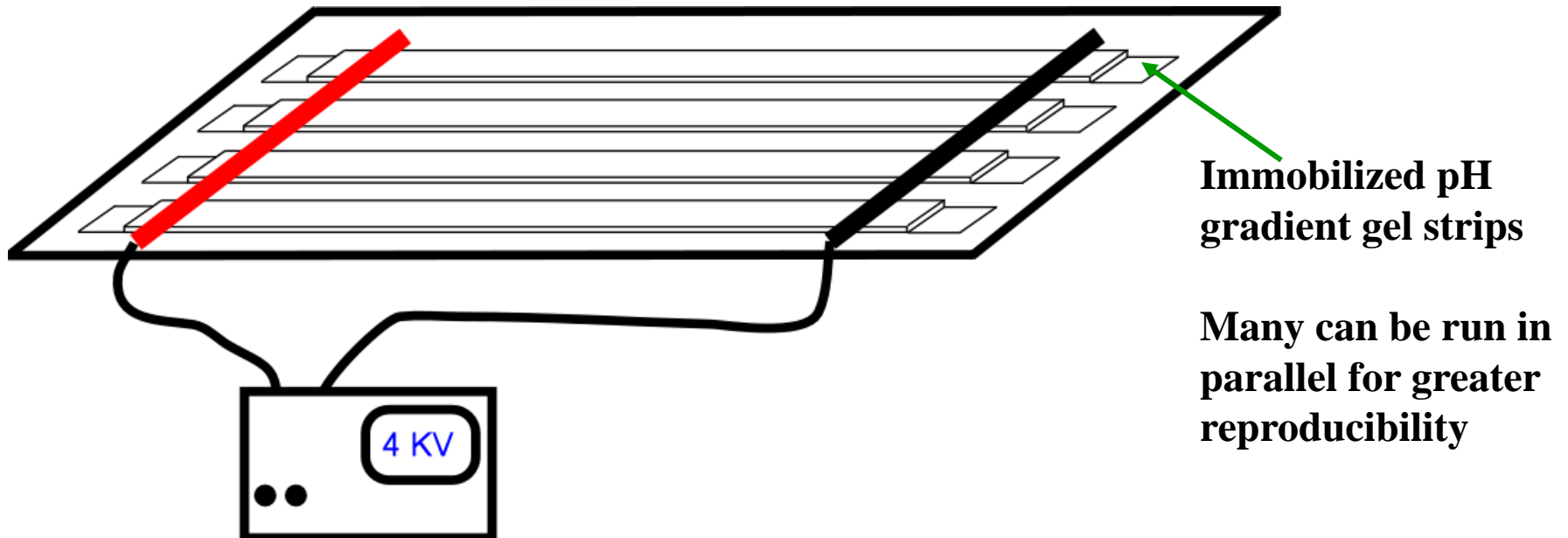
# Isoelectric Focusing

In a pH gradient, under an electric field, a protein will move to the position in the gradient where its net charge is zero.

An immobilized pH gradient is created in a polyacrylamide gel strip by incorporating a gradient of acidic and basic buffering groups when the gel is cast.

Proteins are denatured, reduced, and alkylated, and loaded in a visible dye. The sample is soaked into the gel along its entire length before the field is applied.

Resolution is determined by the slope of the pH gradient and the field strength.



# Polyacrylamide Gel Electrophoresis

**Polyacrylamide gels may have many different constant or gradient compositions.**

**Proteins may be run directly, or are denatured, reduced, and alkylated.**

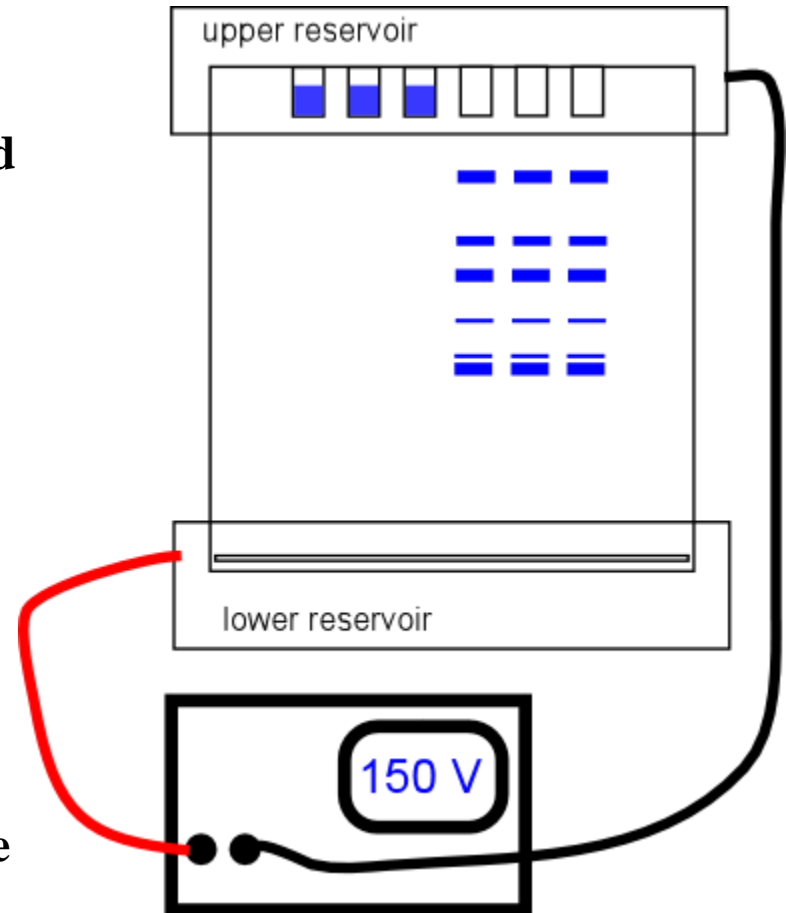
**Samples, with a visible dye added, are loaded in wells cut into the top of the gel.**

**Loading capacity depends on gel size and thickness.**

**In 2D IEF/PAGE, the gel strip from IEF is loaded into a single large well.**

**Molecular weight standards are often run to calibrate the gel.**

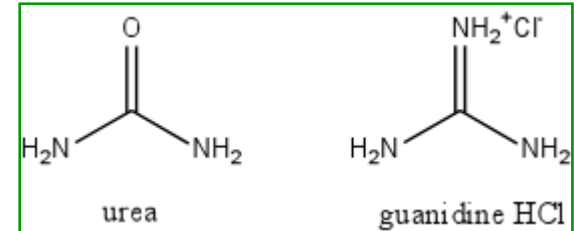
**After separation, the gel is removed from the rig and stained, or bands are blotted onto a membrane.**



# Chemical Methods for Protein Characterization

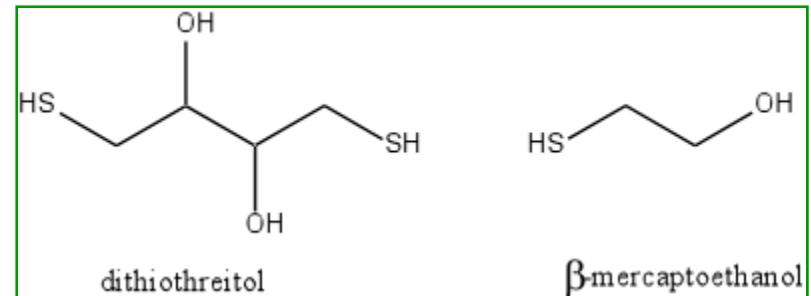
**Denaturation-** dissociates and unfolds proteins

Chaotropes: Urea, Guanidine



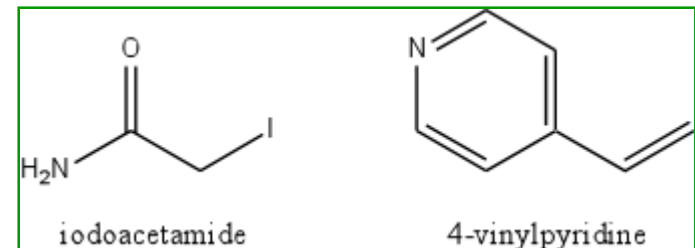
**Disulfide Bond Cleavage**

Reducing Agents: Dithiothreitol, beta-mercaptoethanol



**Cysteine Alkylation-** prevents reoxidation to form disulfides

Alkylating Agents: Iodoacetamide, Vinylpyridine



# Chemical Methods for Protein Characterization: Proteolysis

## Chemical Methods:

**Acid Hydrolysis, various [H<sup>+</sup>], time, temp**

**Cyanogen Bromide cleavage, C-term to Methionine gives a homoserine lactone at Met**

## Enzymes:

<b>Trypsin</b>	<b>C-term to Lys, Arg</b>	<b>pH 8.5</b>
<b>Chymotrypsin</b>	<b>C-term to Y, F, W, H, L</b>	<b>pH 8.5</b>
<b>S. aureus V8 protease</b>	<b>C-term to Glu</b>	<b>pH 8</b>
<b>S. aureus V8 protease</b>	<b>C-term to Glu, Asp</b>	<b>pH 5</b>
<b>Achromobacter protease (Lys-C)</b>	<b>C-term to Lys</b>	<b>pH 8</b>
<b>Arg-C</b>	<b>C-term to Arg</b>	<b>pH 8</b>
<b>Asp-N</b>	<b>N-term to Asp</b>	<b>pH 8</b>
<b>Thermolysin</b>	<b>N-term to L, I, M, F, W</b>	<b>pH 8.5</b>

**Specificity of Proteolysis is a very powerful weapon**

**Specificity of these methods is variable: some excellent, some almost none**

**Ladder Sequencing, sometimes done using MALDI-TOF/MS**

**Manual Edman Degradation, Carboxypeptidase Y digestion, Acid Hydrolysis**

# Chemical Methods for Protein Characterization: A Basic Protocol for Denaturation & Proteolysis

## 1. TCA Precipitation: (if [protein] is $>0.05$ mg/mL)

Chill protein in a microcentrifuge tube to  $0^{\circ}\text{C}$

Add 1/9 volume of cold 100% w/v TCA. Vortex

Incubate at  $0^{\circ}\text{C}$  for 30-60 min

Spin down in microcentrifuge. Remove supernatant

Wash pellet 3x w/ 200  $\mu\text{L}$  cold acetone (do not vortex)

Air dry pellet  $>30$  min

## 2. Redissolving the sample in a chaotrope:

Redissolve protein in 50  $\mu\text{L}$  of fresh 8 M urea/0.4 M Amm. Bicarb.

For efficient digestion, [protein] of  $>0.025$   $\mu\text{g}/\text{mL}$  is required.

(Final volume = 4x the volume of urea added)-adjust as necessary

## 3. Reduction and alkylation of cysteines:

Add 5  $\mu\text{L}$  (or 1/10 volume) of 45 mM DTT

Incubate at  $50^{\circ}\text{C}$  for 15 min. Cool to room temperature

Add 5  $\mu\text{L}$  of 100 mM iodoacetamide

Incubate in the dark at room temperature for 15 min

# Chemical Methods for Protein Characterization: A Basic Protocol for Denaturation & Proteolysis

## 4. Trypsin digestion:

Add 140  $\mu\text{L}$  of ddH<sub>2</sub>O, vortex. Check that pH is between 7.5 and 8.5.

Trypsin added should be a 1:25 weight:weight ratio of protease to sample

Concentration of trypsin should be such that 1 to 5  $\mu\text{L}$  is added to sample

Incubate at 37°C for 24 h. Stop digest by freezing

## Notes:

Trypsin used should be treated with TLCK to inhibit residual chymotrypsin.

Trypsin is made up at 1 or 5 mg/mL in 1 mM HCl.

Aliquots can be stored frozen for up to 3 mos (use once and discard).

This protocol can be used for chymotrypsin or Achromobacter protease (Lys-C)

Final [urea] of 1 M is suitable for Staph. Aureus V-8 protease (Glu-C) or Asp-N protease

In the case of Asp-N a 1:50 to 1:100 w:w ratio should be used; digest for 16 h

# Chemical Methods for Protein Characterization: In-Gel Proteolysis

## Why In-Gel Digestion Works:

1. The gel piece behaves like a sponge. It shrinks and swells in response to addition of aqueous or organic solvent. A gel piece shrunk with organic solvent will suck in an aqueous buffer containing reagents, thus bringing them “inside” the matrix to access the protein.
2. The intact protein is trapped in the gel, so many chemical steps can be performed without significant loss.
3. Many of the peptides resulting from proteolysis within the gel freely diffuse out of the matrix.



# Chemical Methods for Protein Characterization: In-Gel Proteolysis

## 1. Destaining the Gel Band:

Mince gel spot or band with razor blade (cut into 1 mm cubes).

Wash gel pieces with 50% acetonitrile in 50 mM Ammonium bicarbonate pH 8.0

0.5 – 1.0 ml depending on the volume of gel pieces; wash 20 min. on rotator.

If volume of pieces is large (greater than 500 ul) do 2 washes at 20 min each.

Shrink pieces with neat acetonitrile.

Dry in speed vac.

# Chemical Methods for Protein Characterization: In-Gel Proteolysis

## 2. Reduction and alkylation:

Buffer: 6 M guanidine HCl  
0.5 M ammo. Bicarb. PH 8.0  
5 mM EDTA

To 200 ul of above buffer, add 2 ul of 2 M DTT & 0.5 ul 2- or 4-vinyl pyridine

Add just enough to cover the re-swollen gel pieces  
Incubate 15 min at 37C or 30 min RT

Remove excess reduction and alkylation mix; wash gel pieces 2 times with 1 ml aliquots of 50 mM ammonium bicarbonate pH 8.0.

Shrink with neat acetonitrile. Swell in 50 mM AmBic pH 8.0.

Shrink with neat acetonitrile. Swell in 50 mM AmBic pH 8.0.

Shrink with neat acetonitrile. Dry in speedvac.

# Chemical Methods for Protein Characterization: In-Gel Proteolysis

## 3. Proteolysis:

Swell in 50 mM AmBic pH 8.0 containing 12.5 ng/ul trypsin.

Add enough trypsin solution to cover fully swollen gel pieces

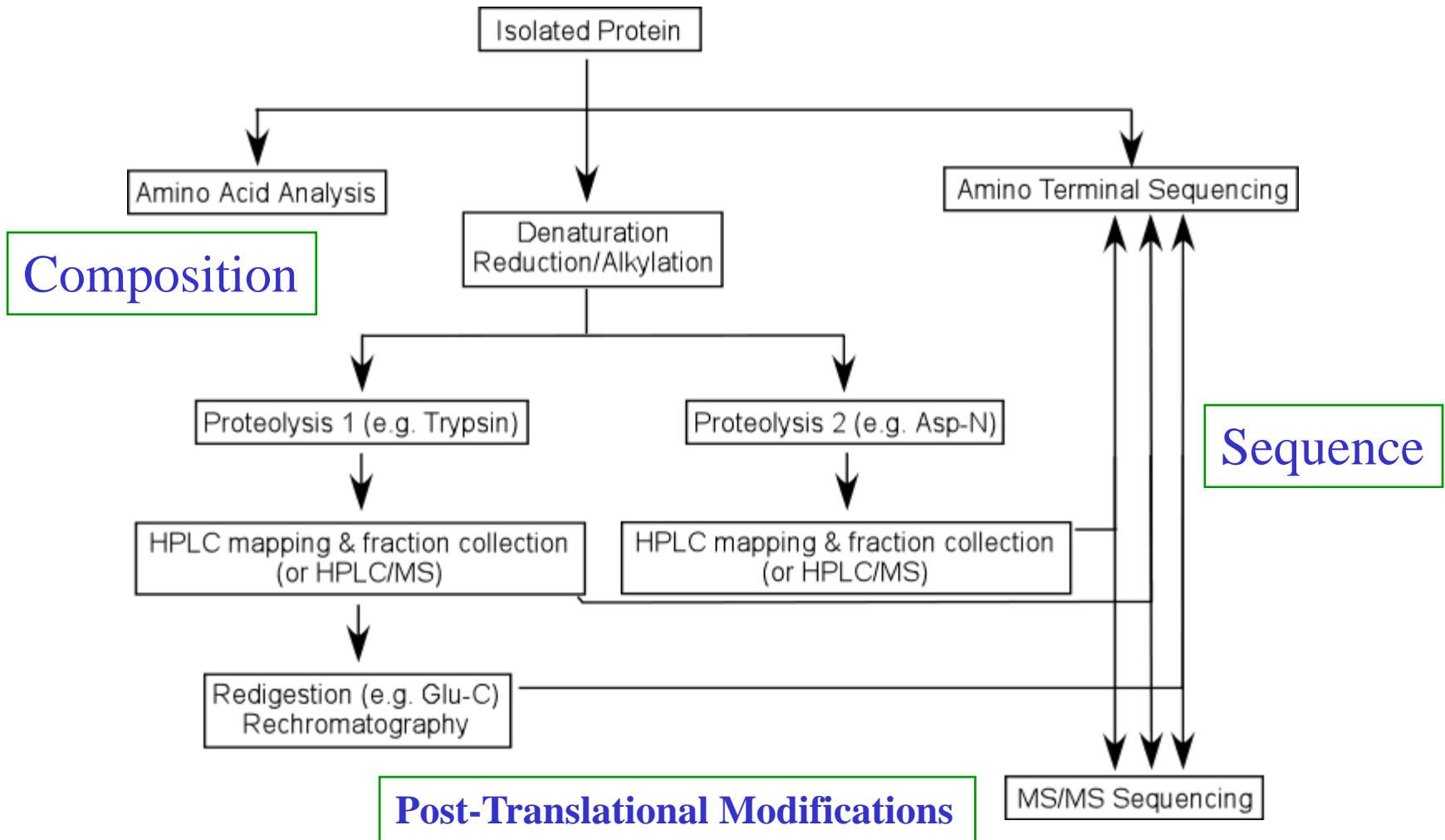
Digest for 16 – 18 hours.

## 4. Analysis:

For MALDI-TOF/MS: an aliquot may be taken from the supernatant around the gel pieces

For LC/MS: all the volume plus an additional extraction of the pieces in 50 mM AmBic pH 8.0 may be combined

# Combining Analytical Methods for Protein Characterization



Full coverage almost always requires multiple digests.  
Identification essentially never does.