Fundamentals of Protein Chemistry

Amino Acid and Peptide Chemistry

Transcription and Translation

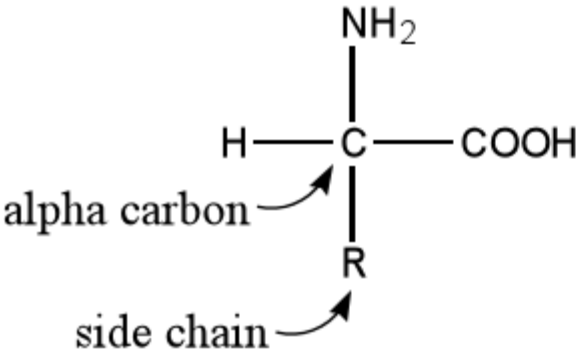
Post-Translational Modifications

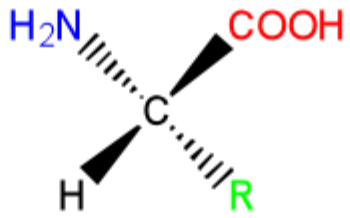
Classical Analytical Methods

In-Gel Proteolysis

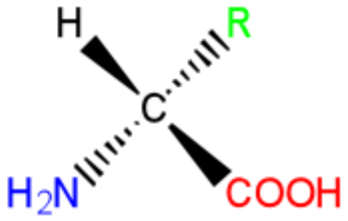
Amino Acids are the Basic Structural Units of Proteins

General Structure of an Amino Acid

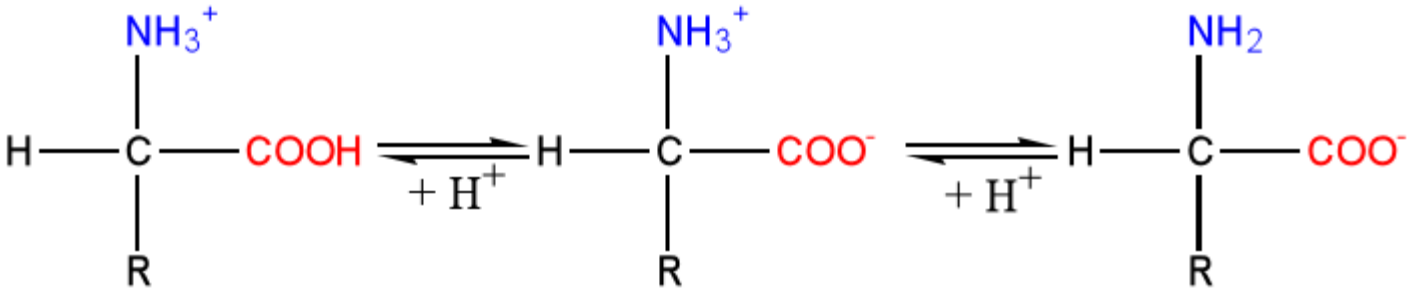




L-Isomer



D-Isomer



pH 1 pH 7 pH 11

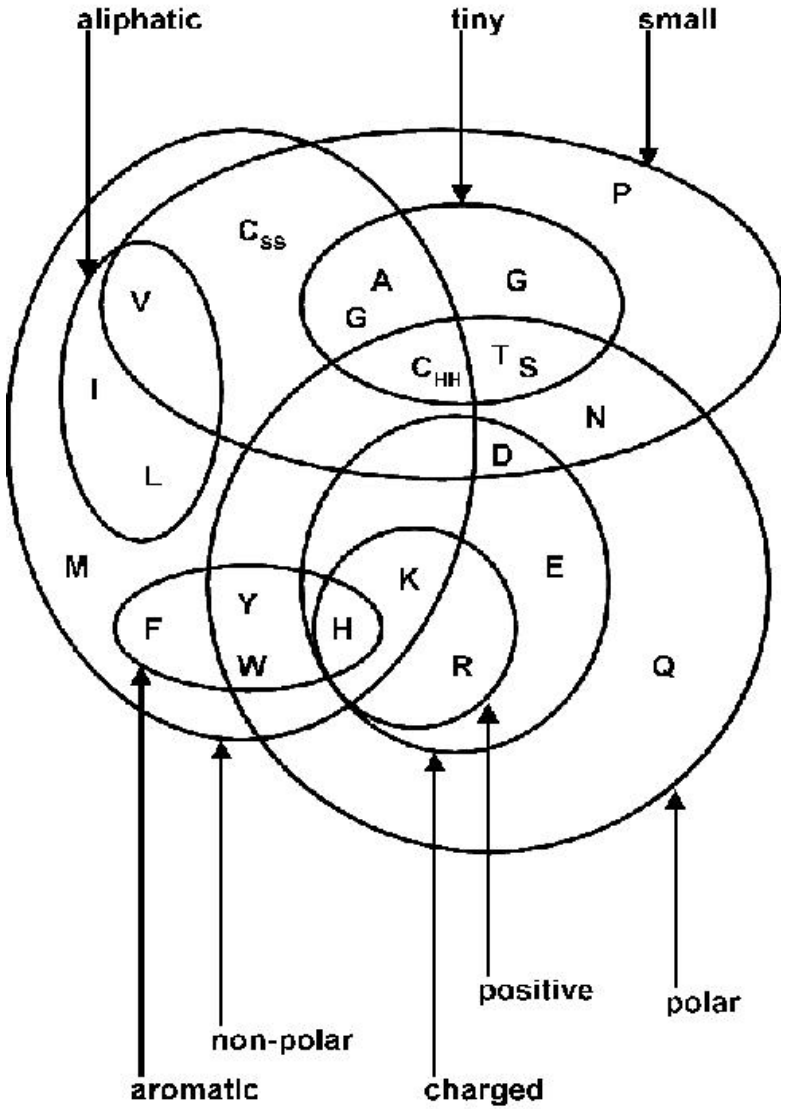
The Ionization State of Amino Acids Changes with pH

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

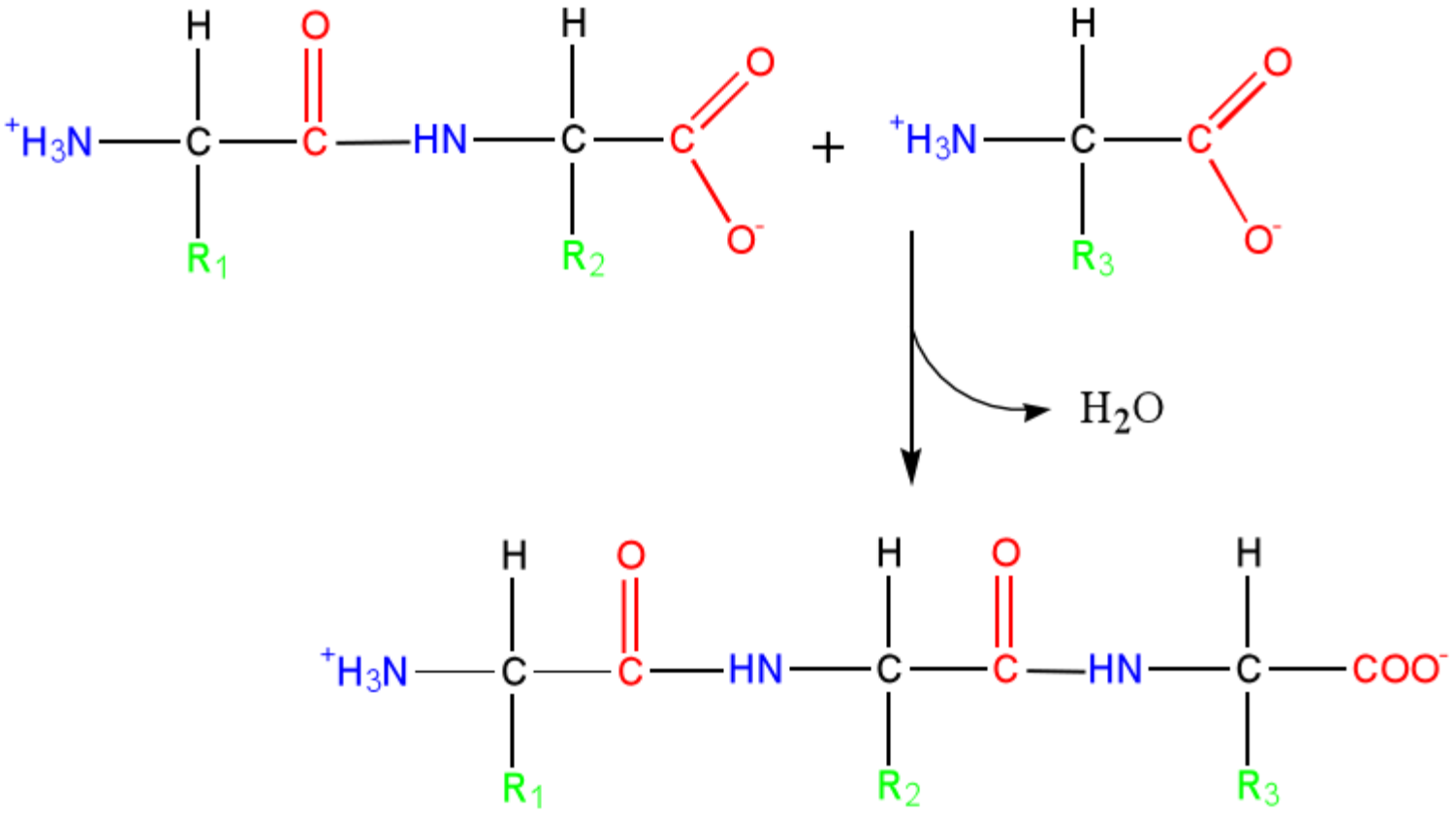
The Twenty Amino Acid Side Chains Vary Significantly

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



http://www.imb-jena.de/image_library/GENERAL/aa/chemprop.jpg

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.

pH = pK + log [A-]/[HA]

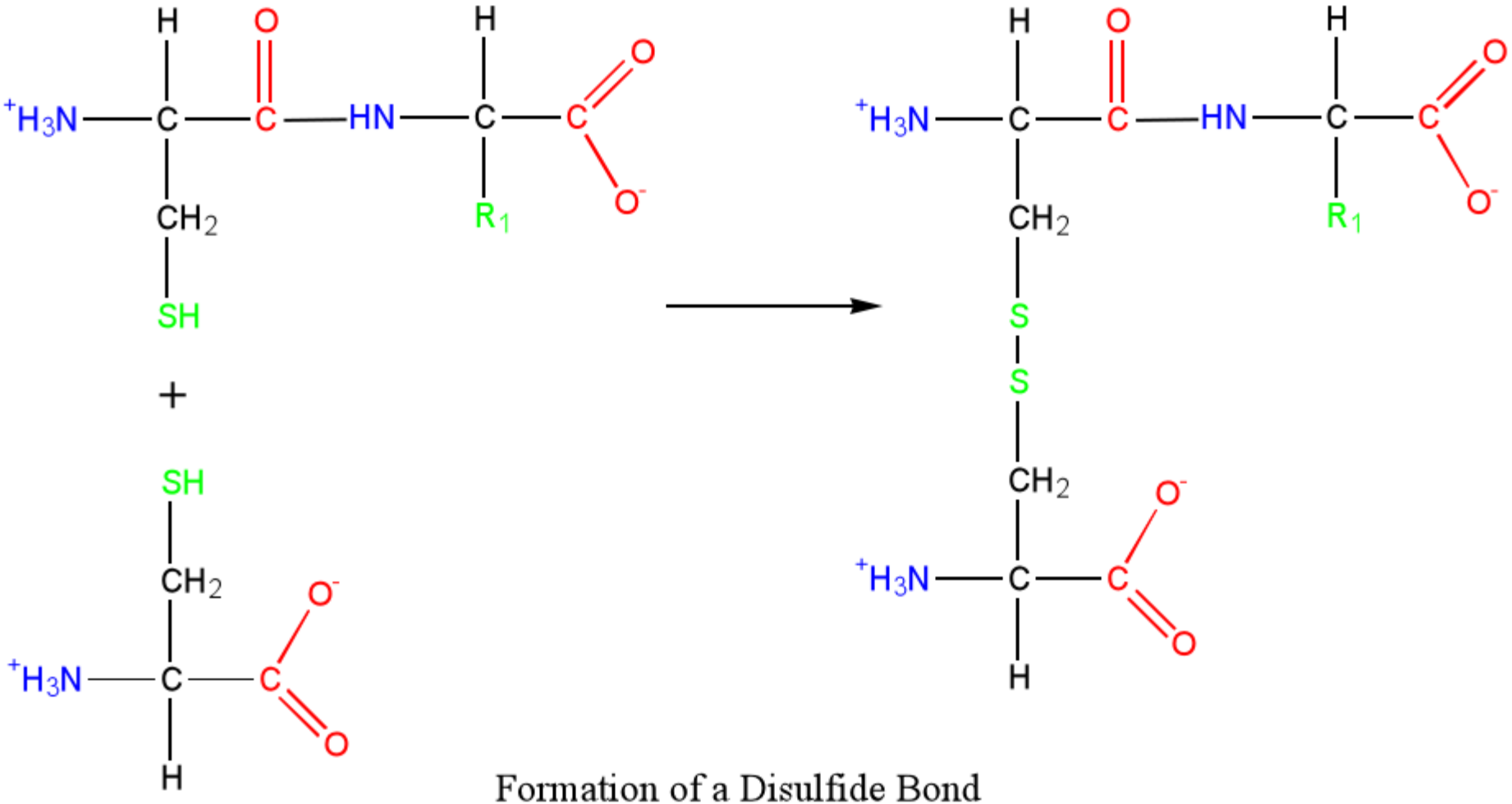
pK of alpha-COOH groups:	**1.8 - 2.4**
pK of alpha-NH2 groups:	**9.0 - 10.8**
pK of ionizable side chains:	**3.9 - 12.5**

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



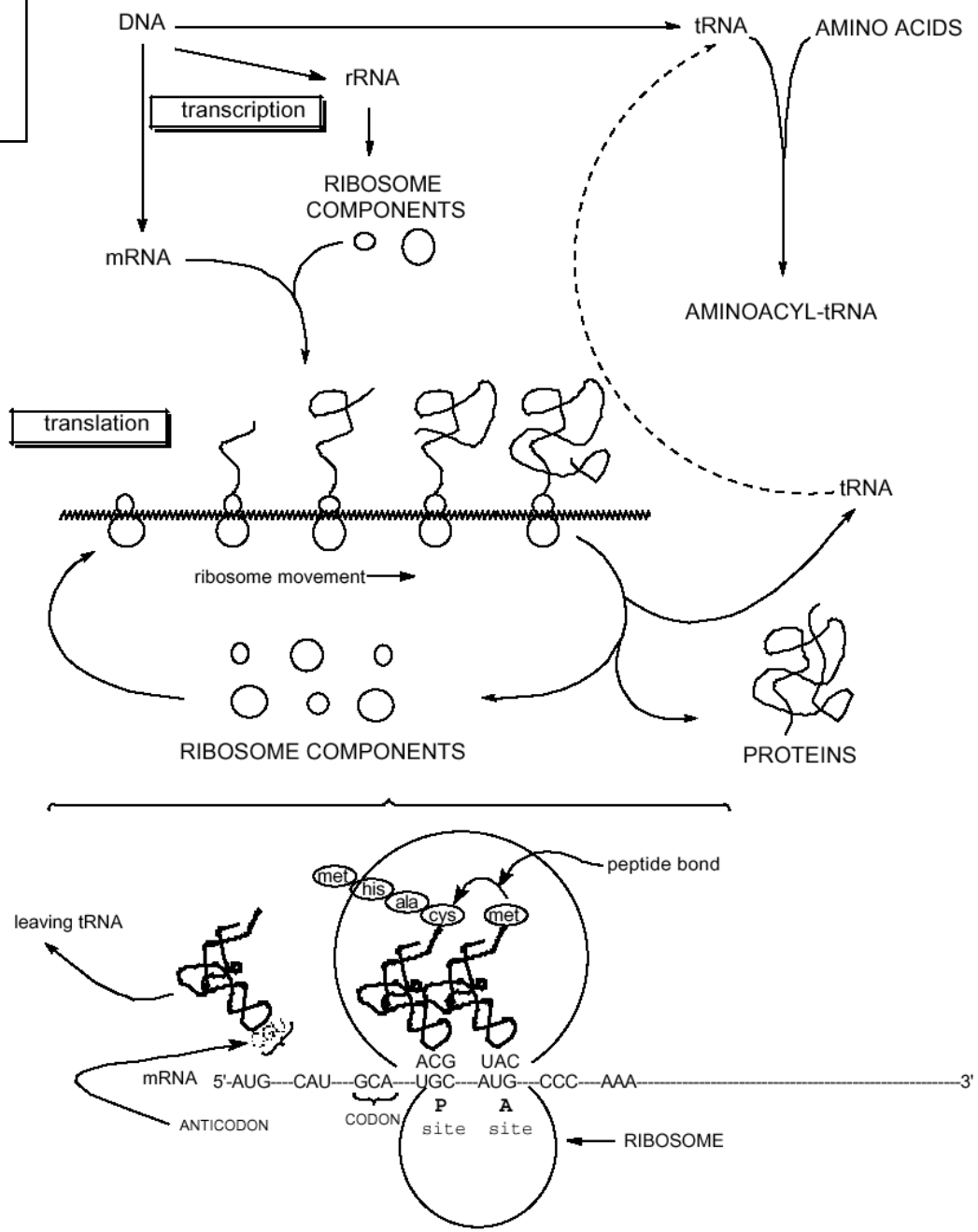
Formation of a Disulfide Bond

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.

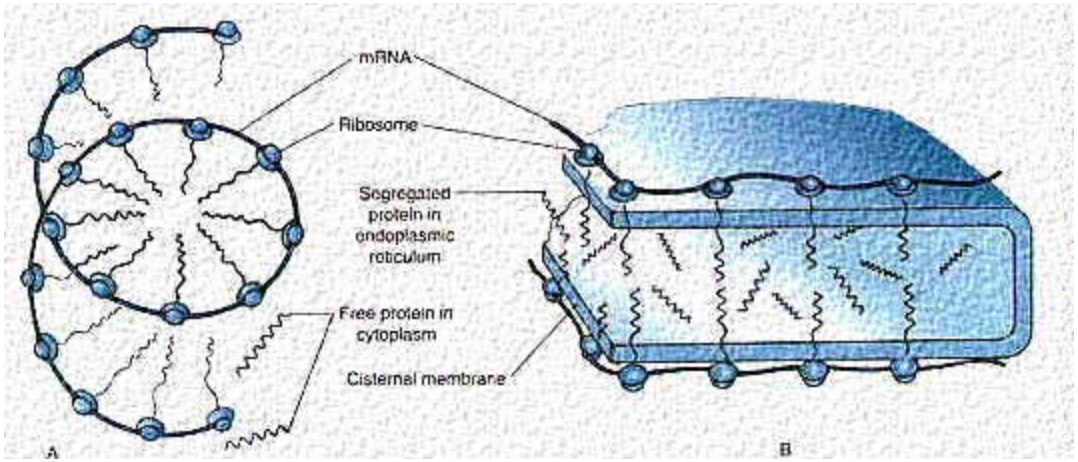


T. Zamis, U. Wisc., Stephens Point
http://chemdept.uwsp.edu/tzamis/Chem365Biochemistry.html

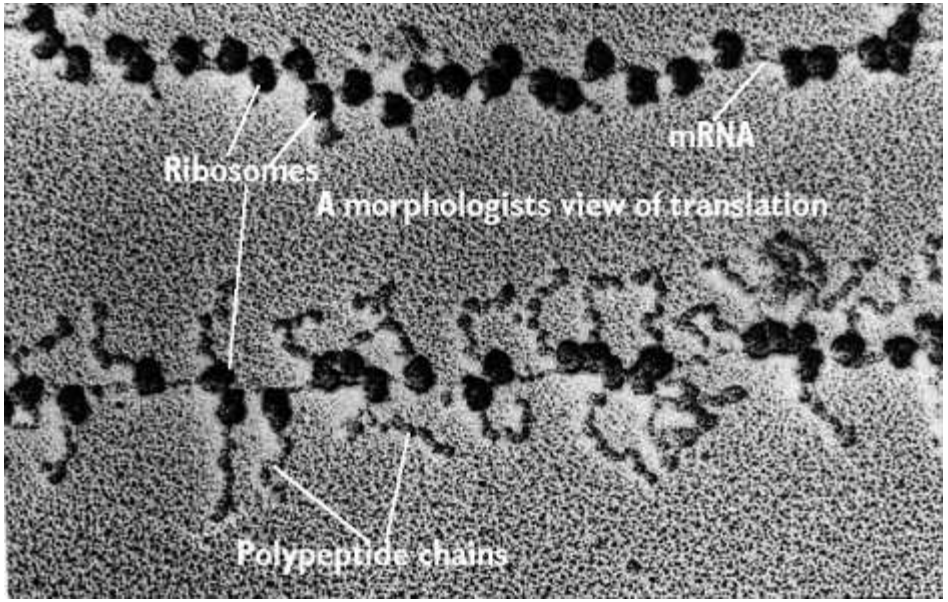
Translation: Amino Acid Codons

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

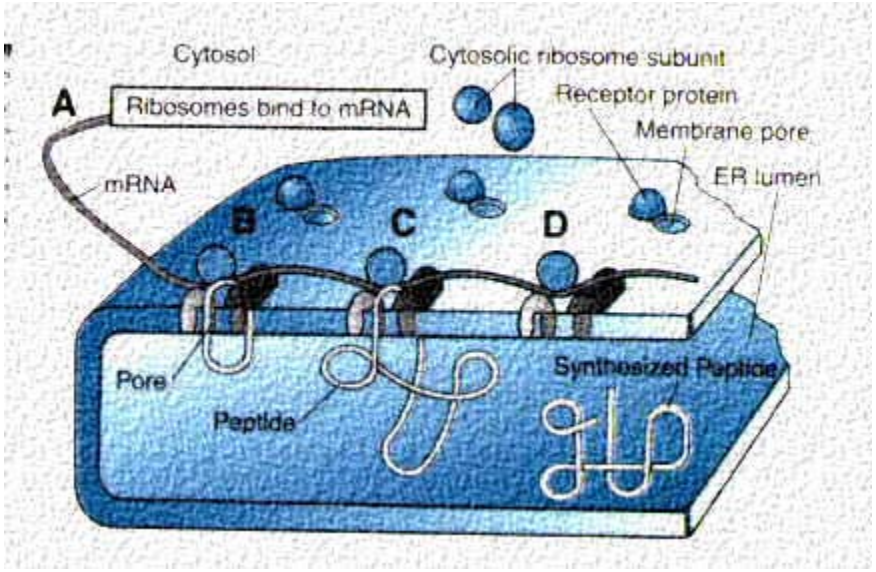


Molecular Biology of the Cell by Bruce Alberts, Dennis Bray, Julian Lewis, Martin Raff, Keith Roberts, and James D. Watson, Garland Publishing, NY 1994

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.



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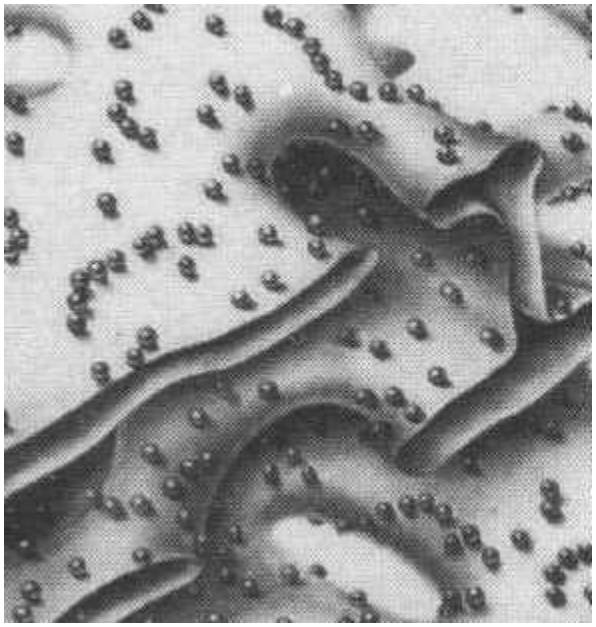
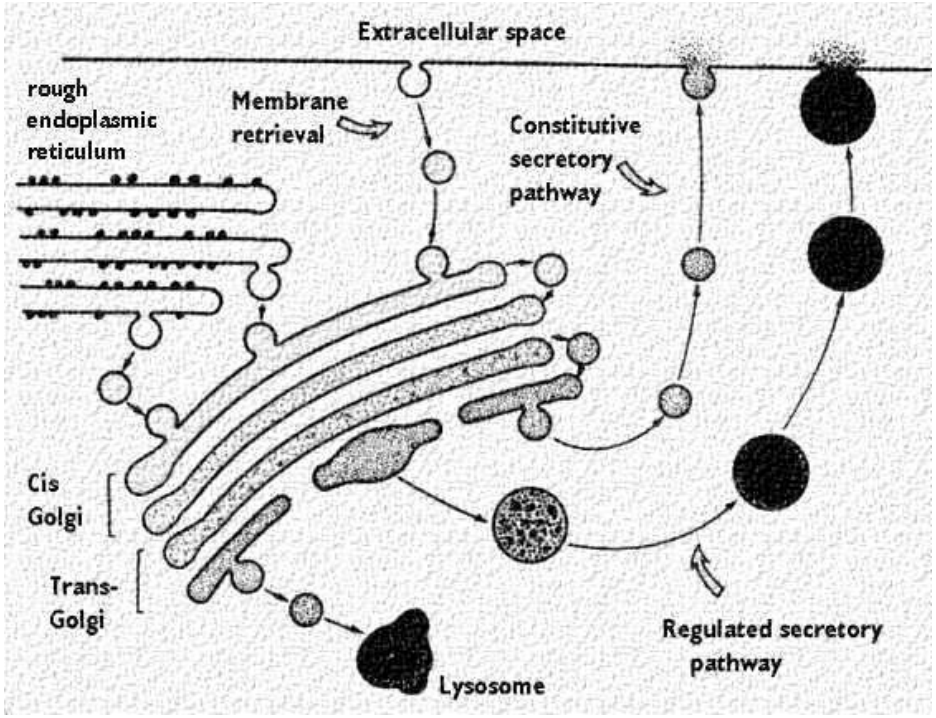


A Textbook of Histology by D Fawcett. Chapman and Hall, N.Y. 1994

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.



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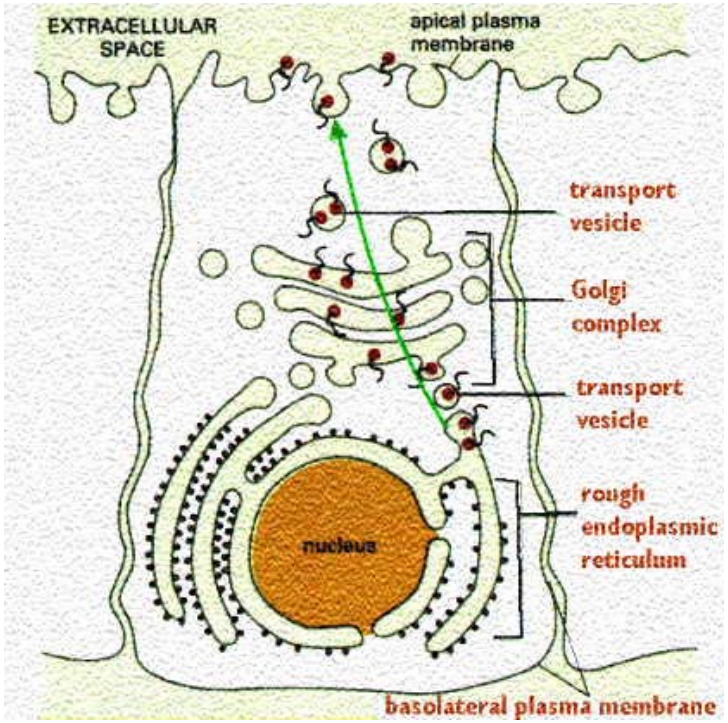

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Protein Post-Translational Modification

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_2-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

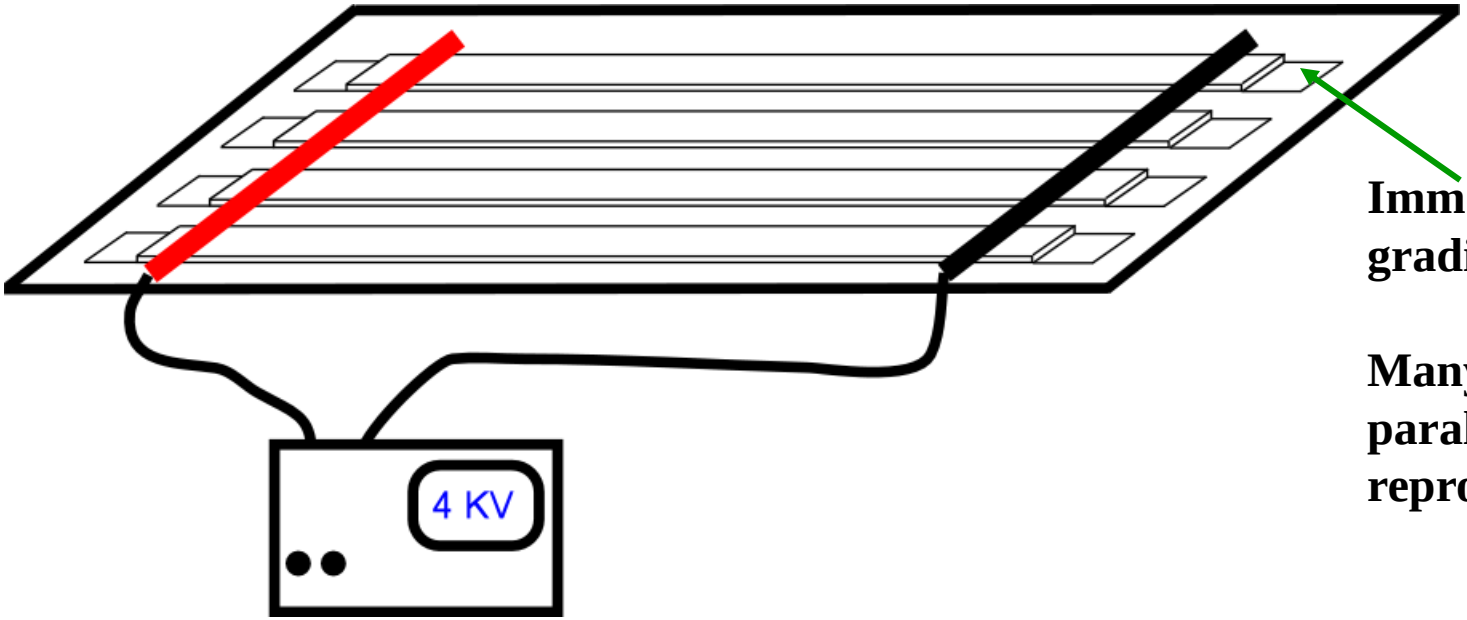
Isoelectric Focusing

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.



Immobilized pH gradient gel strips

Many can be run in parallel for greater reproducibility

Polyacrylamide Gel Electrophoresis

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

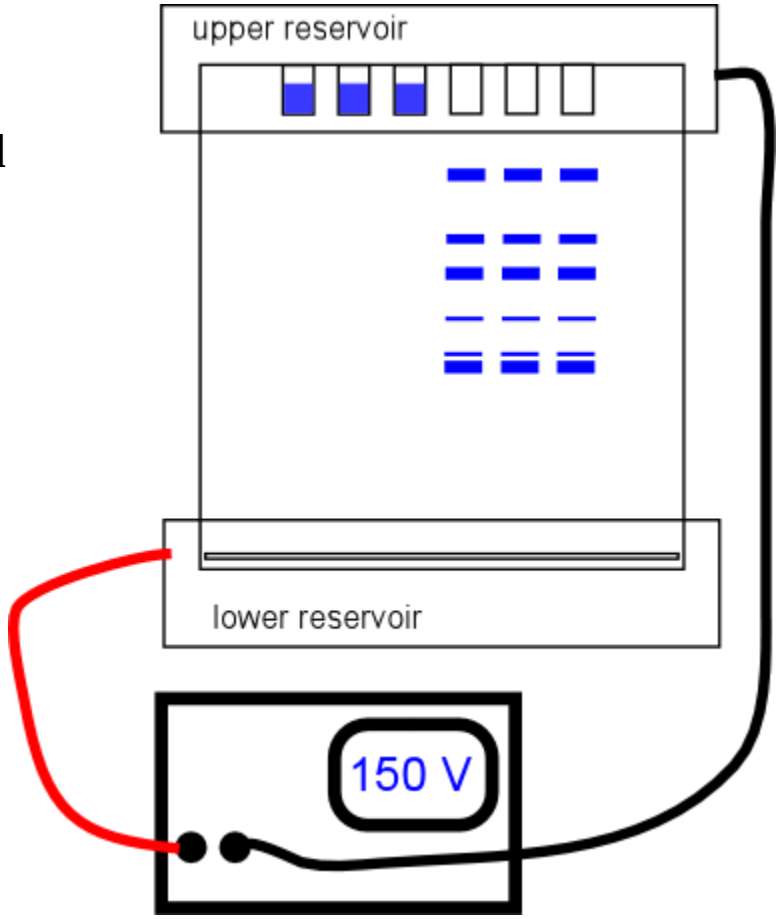
Proteins may be run directly, or are denatured, reduced, and alkylated. Samples, with a visible dye added, are loaded in wells cut into the top of the gel.

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.

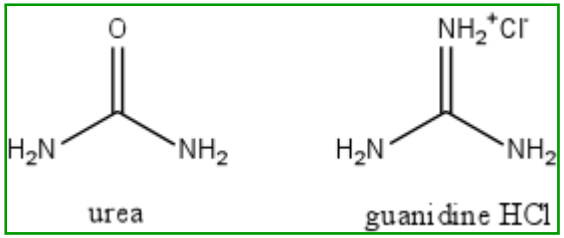
After separation, the gel is removed from the rig and stained, or bands are blotted onto a membrane.



Chemical Methods for Protein Characterization

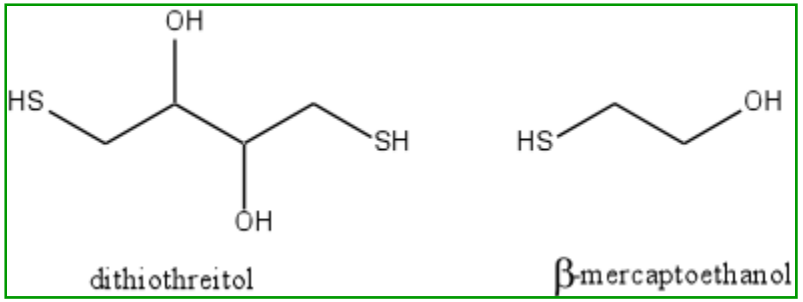
Denaturation- dissociates and unfolds proteins

Chaotropes: Urea, Guanidine



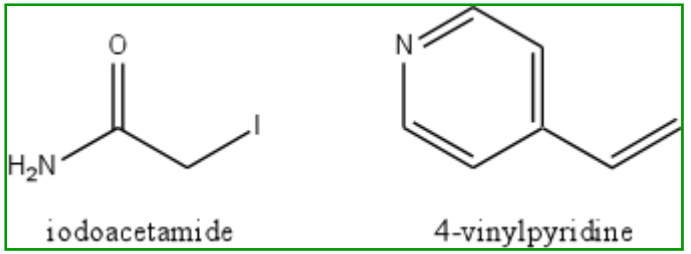
Disulfide Bond Cleavage

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+], time, temp

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

Enzymes:

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

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°C
Add 1/9 volume of cold 100% w/v TCA. Vortex
Incubate at 0°C for 30-60 min
Spin down in microcentrifuge. Remove supernatant
Wash pellet 3x w/ 200 µL cold acetone (do not vortex)
Air dry pellet >30 min

2. Redissolving the sample in a chaotrope:

Redissolve protein in 50 µL of fresh 8 M urea/0.4 M Amm. Bicarb.
For efficient digestion, [protein] of >0.025 µg/mL is required.
(Final volume = 4x the volume of urea added)-adjust as necessary

3. Reduction and alkylation of cysteines:

Add 5 µL (or 1/10 volume) of 45 mM DTT
Incubate at 50°C for 15 min. Cool to room temperature
Add 5 µL of 100 mM iodoacetamide
Incubate in the dark at room temperature for 15 min

Barbara Merrill, Bayer Corp.

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

4. Trypsin digestion:

Add 140 μL of ddH_2O, 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 μL 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

Barbara Merrill, Bayer Corp.

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.

Mary B. Moyer, GlaxoSmithKline, after "Femmtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry", M. Wilm, A. Shevchenko, T. Houthaeve, S. Breit, L. Schweigerer, T. Fotsis, M. Mann; Nature, 1996, 466-469.

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.

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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.

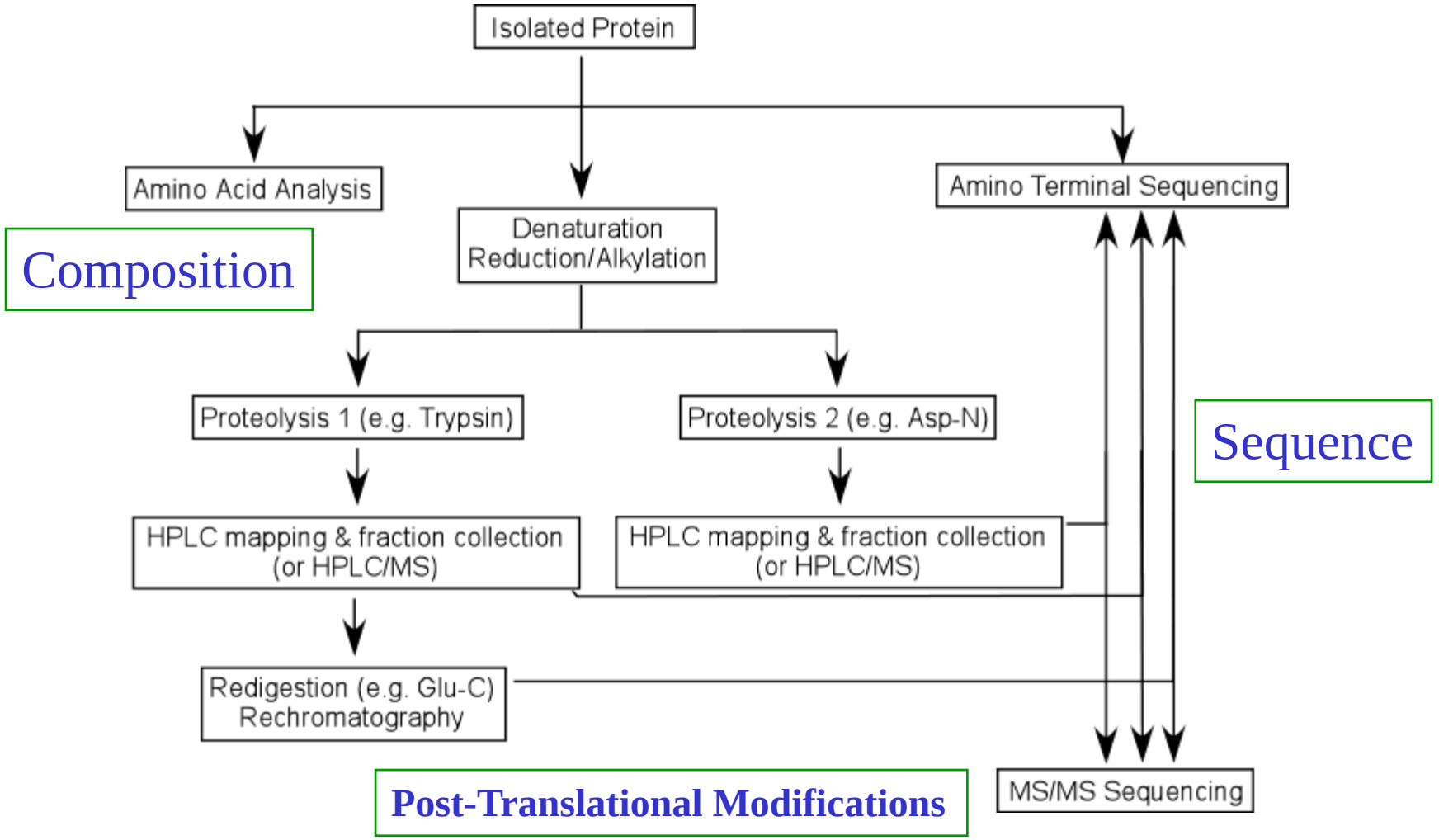
4. Analysis:

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

Mary B. Moyer, GlaxoSmithKline, after "Femmtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry", M. Wilm, A. Shevchenko, T. Houthaeve, S. Breit, L. Schweigerer, T. Fotsis, M. Mann; Nature, 1996, 466-469.

Combining Analytical Methods for Protein Characterization



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