# Non-enzymatic Protein, Function and Protein Analysis

## Cellular Functions

### Structural Proteins

- Primary structural proteins in the body are collagen, elastin, keratin, actin and tubulin
- These proteins have highly repetitive organization (**motif**)
- Organization gives most structural proteins a fibrous nature

#### Collagen

- Trihelical fiber: three left handed helices woven together to form a secondary right-handed helix)
- Makes up most of the extracellular matrix of connective tissue
- Important in providing strength and flexibility

#### Elastin

- Primary role is to stretch and recoil like a spring so that it restores the original shape of the tissue
- Component of the extracellular matrix

#### Keratins

- Intermediate filament proteins found in epithelial cells
- Contribute to mechanical integrity of the cell
- Also function as regulatory proteins
- Primary protein that makes up hair and nails

#### Actin

- Makes up microfilaments and the thin filaments in myofibrils
- Most abundant protein in eukaryotic cells
- Polar proteins: having a positive and negative side allows motor proteins to travel in one direction along an Actin filament

#### Tubulin

- Makes up microtubules
    - Microtubules provide: structure; chromosome separation; and intracellular transport with kinesin and dynein
- Has polarity: negative end is usually located near the nucleus while the positive end is located in the periphery of the cell

### Motor Proteins

- Some structural proteins have motor function in the presence of proteins
    - E.g. – motile cilia in bacteria or the flagella in sperm
- Enzymatic Activity: act as **ATPases** which powers the conformational change necessary for motor function
- Motor proteins interact either with actin or microtubules

#### Myosin

- Primary motor proteins that interacts with actin
- Thick filament in a myofibril and is also involved in cellular transport

- Each subunit has a head and a neck
    - Movement of the neck is responsible for the power stroke of sarcomere contraction.

### Kinesins and Dyneins

- Motor proteins associated with microtubules
- Have two heads, at least one stays attached to tubulin at all times
- Kinesin: play a role in aligning chromosomes during metaphase and depolymerizing microtubules during anaphase of mitosis.
- Dyneins: involved in the sliding movement of cilia and flagella
- Both proteins are important for vesicle transport in the cell
    - Kinesins bring vesicles towards the positive end of the microtubule
    - Dyneins bring vesicles towards the negative end of the microtubule

## Binding Proteins

- Classified by proteins that have a stabilizing function in individual cells of the body. These proteins transport or sequester molecules by binding to them
- E.g. – hemoglobin, calcium-binding proteins, DNA-binding proteins
    - Each binding protein has an affinity curve for its molecule of interest
        - If goal of protein is sequestration: binding protein will usually have a high affinity over a long range of conditions in order to keep the target molecule bound at nearly 100%
        - If goal of protein is transport: varying affinity depending on environmental conditions so that equilibrium concentrations can be maintained

## Cell Adhesion Molecules

- Proteins found on the surface of most cells
- Aid in binding the cell to the extracellular matrix or other cells
- Are all integral membrane proteins

### Cadherins

- Group of glycoproteins that mediate calcium-dependent cell adhesion
- Hold similar cell types together, each cells usually have type-specific cadherins

### Integrins

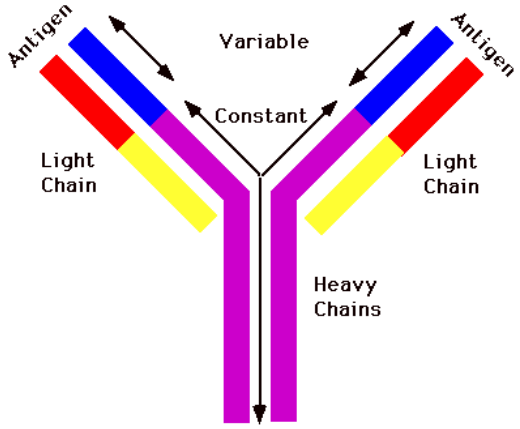
- Group of proteins that have two membrane-spanning chains called $\alpha$ & $\beta$.
    - Chains are important in binding to and communicating with the extracellular matrix
- Play an important role in cellular signaling and can greatly impact cellular function by promoting cell division, apoptosis, or other processes

### Selectins

- Bind to carbohydrate molecules that project from other cell surfaces
- Weakest bonds formed by CAMs
- Expressed on white blood cells and endothelial cells that line blood vessels
- Play an important role in **host defense**: including inflammation and white blood cell mitigation

## Immunoglobulins

- Most prominent type of protein found in the immune system is the antibody (or immunoglobulins (Ig))
- Antibodies: proteins produced by B-cells that function to neutralize targets in the body, such as toxins and bacteria, and then recruit other cells to help eliminate the threat.
    - Y-shaped proteins that are made up of two identical heavy chains
    - Disulfide linkage and noncovalent interaction hold the heavy and light chains together
        - Each antibody has an **antigen-binding region** at the tips of the "Y"
            - Specific polypeptide sequences that will bind **one** specific antigenic sequence.
        - Remaining part of antibody is the **constant region**
            - Involved in the recruitment and binding of other cells of the immune system.
- When antibodies bind to their targets (antigens), can cause one of three outcomes:
    - Neutralize the antigen which makes the pathogen or toxin unable to exert its effect on the body
    - Opsonization: marking the pathogen for destruction by other white blood cells immediately
    - Agglutinating: clump together the antigen and antibody into a large insoluble protein complexes that can be phagocytized and digested by macrophages




# Biosignaling

- Process by which cells receive and act on signals
- Proteins act as extracellular ligand, transporters for facilitated diffusion, receptor proteins and second messengers
- Can have functions involved in substrate binding or enzymatic activity

## Ion Channels

- Proteins that create specific pathways for charged molecules
- All permit facilitated diffusion of charged molecules
    - **Facilitated Diffusion:** diffusion of molecules down a concentration gradient through a pore in the membrane created by a transmembrane protein.

    - Used for molecules that are impermeable to the membrane (large, polar or charged)
    - Allows integral membrane proteins to serve as channels for these substrates to avoid the hydrophobic fatty acid tails of the phospholipid bilayer
- $K_m$ & $v_{max}$ paramters that apply to enzymes can also apply to transporters (ion channels)
    - $K_m$ refers to the solute concentration at which the transporter is functioning at half of its maximum capacity

### Ungated Channels

- Unregulated since they have no gates
    - E.g. – potassium ion channel.
- Will always be movement unless specified ion is at equilibrium

### Voltage-Gated Channels

- Gate is regulated by the membrane potential change near the channel
    - E.g. – voltage-gated sodium channels
- Channels are closed under resting conditions; depolarization of the cell membrane leads to a conformational change in the protein that allows them to quickly open.

### Ligand-Gated Channels

- Binding of a specific substrate or ligand to the channel causes it to open or close
    - E.g. – neurotransmitters act at the postsynaptic membrane. GABA (inhibitory N.T) binds to the chloride channel and opens it.

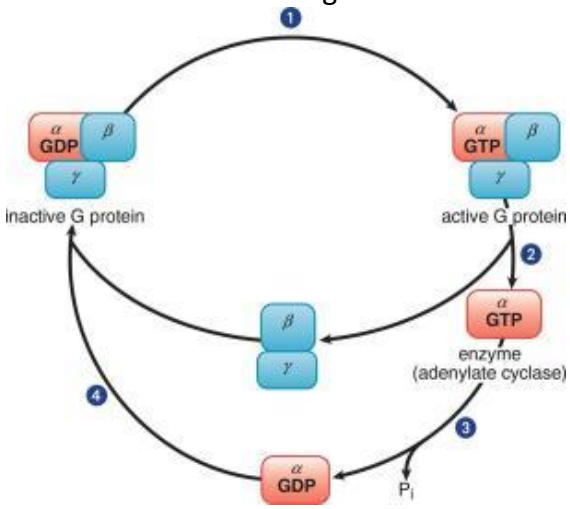
### Enzyme-Linked Receptors

- Membrane receptors that display catalytic binding in response to ligand binding
- Have three primary protein domains
    - Membrane-Spanning: anchors the receptor in the cell membrane
    - Ligand-Binding: stimulated by the appropriate ligand and induces a conformation change that activates the **catalytic domain.**
    - Catalytic: Often results in the initiation of a **second messenger cascade**
- E.g. – Receptor tyrosine kinases (RTK)

## G Protein-Coupled Receptors (GPCR)

- Family of integral membrane proteins involved in signal transduction
- Seven membrane spanning alpha-helices
    - Receptors differ in specificity of the ligand-binding area found on the extracellular surface of the cell
- Heterotrimeric G Proteins: how the GPCRs transmit signals to an effector in the cell
    - Named for their intracellular link to guanine nucleotides (GDP & GTP)
    - Binding of ligand increases the affinity of the receptor for the G protein
    - Binding of G-protein represents a switch to the active state and affects the intracellular signaling pathway
    - G-proteins can result in either stimulation or inhibition, three main types:
        - $G_s$: stimulated adenylate cyclase which increase levels of cAMP in the cell
        - $G_i$: inhibits adenylate cyclase which decreases levels of cAMP in the cell

        - $G_q$: activates *phospholipase C* – cleaves a phospholipid from the membrane to form $PIP_2$.
            - $PIP_2$ is then cleaved into DAG and $IP_3$
                - $IP_3$ can open calcium channels in the endoplasmic reticulum which increases calcium levels in the cell
- G-Proteins have three subunits: $\alpha$, $\beta$, and $\gamma$
    - In inactive form, the alpha-subunit binds GDP and is in a complex with the other two subunits
    - When a ligand binds to the GPCR, the receptor becomes activated and engages the corresponding G protein
        - Causes the conversion of GDP to GTP which allows the alpha subunit to dissociate from the other two subunits
    - Alpha subunit alters the activity of ***adenylate cyclase*** (either inhibitory or stimulating)
    - Once GTP is dephosphorylated to GDP, the alpha subunit binds to the other two and the G protein is made inactive again.




## Protein Isolation

- Proteins and other biomolecules are isolated from body tissue or cell cultures by cell lysis or **homogenization:** crushing, grinding or blending the tissue of interest into an evenly mixed solution.
- Centrifugation: can isolate proteins from much smaller molecules after homogenization
    - Precursor to other isolation techniques: electrophoresis and chromatography

### Electrophoresis

- Works by subjecting compounds to an electric field which moves them according to their net charge and size.
    - Negatively charged compounds will migrate towards the positively charged anode, and positively charged compounds will migrate towards the negatively charged cathode

- Migration Velocity: velocity of the migration and is directly proportional to the electric field strength (**E**) and to the net charge of the molecule (z), and is inversely proportional to a frictional coefficient, *f*. $v = \frac{Ez}{f}$
- **Polyacrylamide gel** is the standard medium for electrophoresis
    - Slightly porous matrix mixture that solidifies at room temperature
    - Proteins travel through this matrix in relation to their size and charge
        - Gel allows smaller molecules to pass through easily and retains large particles.
    - A molecule will move faster through the gel if it is small, highly charged or placed in a large electric field

### Native PAGE (Polyacrylamide gel electrophoresis)

- Method for analyzing proteins in their native states.
- This is limited by the varying mass-to-charge and mass-to-size ratios of cellular proteins since different proteins may experience the same level of migration
- Protein can be recovered from the gel if the sample hasn't been stained
- Most useful to compare molecular size or the charge of proteins known to be similar in size

### (Sodium Dodecyl Sulfate) SDS-PAGE

- Separates proteins on the basis of **relative molecular mass alone**.
- SDS disrupts all noncovalent interactions
    - Binds to protein and creates large chains with net negative charges which neutralizes the protein's original charge and denatures the protein
- The only variable that affects the velocity is **E** and *f*

### Isoelectric Focusing

- Proteins can be separated based on their **isoelectric point** (**pI**)
    - pI is the pH at which the protein or amino acid is electrically neutral
    - **Zwitterion** is the neutral form for single amino acids, calculation of this point was done in first chapter
- Exploits the acidic and basic properties of amino acids by separating on the basis of pI.
    - Mixture is placed in a gel with a pH gradient where the anode has acidic gel and is positive, and the cathode has basic gel and is negative, middle is neutral
    - Electric field is then generated across the gel
    - Positively charged proteins will migrate towards the cathode and negatively charged proteins will migrate towards the anode
    - When the protein reaches a portion of the gel that has a pH equal to the its pI, the protein takes on a neutral charge and will stop moving.

## Chromatography

- Require the homogenized protein mixture to be fractionated through a porous matrix
- Allow for the protein to be immediately available for identification and quantification
- Overarching concept: the more similar a compound is to its surroundings (by polarity, charge, etc.), the more it will stick to and move slowly through its surroundings.

- Process begins by placing a sample onto a solid medium called the **stationary phase** or **adsorbent**
    - Mobile phase is then run through the stationary phase
        - Allows the sample to **elute**: run the sample through the stationary phase
    - Components that have high affinity for the stationary phase will barely migrate at all
    - Components that have high affinity for the mobile phase will migrate quickly
    - Retention Time: amount of time a compound spends in the stationary phase
        - Varying retention times of each compound results in the separation of components within the stationary phase (**partitioning**)

### Column Chromatography

- Column is filled with polar silica or alumina beads as an adsorbent, and gravity moves the solvent + compounds down the column
    - The less polar a compound, the faster it will elute (shorter retention time)
        - Solvent polarity can be easily changed by altering the pH or salinity
- Eventually solvent drips out of the column and it can be collected at different intervals to get a specific compound of interest.

### Ion-Exchange Chromatography

- Beads of column are coated with charged substances so that they attract or bind compounds that have an opposite charge
- After all other compounds have moved through the column, a salt gradient can be used to elute the charged molecules that have stuck to the column

### Size-Exclusion Chromatography

- Beads used in the column contain tiny pores of varying sizes which allow small compounds to enter the beads
- This effectively slows down small molecules while allowing larger molecules to elute quickly

### Affinity Chromatography

- Columns can be customized to bind any protein of interest by creating beads with a high affinity for that protein.
    - Can be accomplished by coating the beads with a receptor that binds to the protein or a specific antibody to the protein.
        - Protein is retained in the column
- Once the protein is retained, it can be eluted by washing the column with a free receptor which competes with the bead-bound receptor and ultimately frees the protein from the column
- Alternatively, an eluent can be created with a specific pH or salinity to disrupt the ligand bonds
    - Drawback is that the recovered substance may be bound to the eluent

# Protein Analysis

## Protein Structure

- Can be determined through **X-ray crystallography** and **nuclear magnetic resonance (NMR) spectroscopy**
- X-Ray Crystallography: most reliable and common method
    - Protein must be isolated and crystallized beforehand
    - Measures electron density on an extremely high-resolution scale
    - Generates an X-Ray diffraction pattern, small dots in pattern are then used to determine protein's structure
- NMR is discussed in organic chemistry and accounts for 25% of the protein structure determination.

## Amino Acid Composition

- Can be determined by complete protein hydrolysis and subsequent chromatographic analysis, but actual sequence of amino acids cannot be determined since hydrolysis is a random process.
- If the sequence of amino acids is needed, the protein needs to be sequentially digested with specific cleavage enzymes.
    - **Edman Degradation:** uses cleavage to sequence proteins of up to 50-70 amino acids
        - Selectively and sequentially removes the N-terminal amino acid of the protein which is then analyzed by mass spectroscopy
    - Larger proteins are digested with chymotrypsin, trypsin and cyanogen bromide
        - Selectively cleaves proteins at specific amino acid residues, which creates smaller fragments that can be analyzed using electrophoresis or the Edman degradation
        - Location of disulfide links and salt bridges cannot be determined with this method since those connections are broken

## Activity Analysis

- Protein activity can be determined by monitoring a known reaction with a given concentration of substrate and then comparing it with a standard
- Activity is correlated with concentration but is also affected by the purification methods used
- Most applicable when reactions have a colour change associated with it since the can be quickly identified from a chromatographic analysis

## Concentration Determination

- Determined through spectroscopy. Can be analyzed with UV spectroscopy without any treatment since proteins contain aromatic side chains
    - This analysis is sensitive to sample contaminant
- Another method is to take advantage of the fact that proteins cause colorimetric changes with specific reactions: **bicinchoninic acid (BCA) assay, Lowry reagent assay, and Bradford protein assay.**

## Bradford Protein Assay

- Most common since it is reliable and simple
- Mixes a protein in solution with blue dye
- Dye gives up protons when it binds to amino acid groups and turns blue in the process
- The larger the concentration of blue dye, the higher the concentration of the protein
    - Due to ionic attraction between dye and protein which causes stabilization of the blue dye
- Samples of known concentrations are reacted with the Bradford reagent and the absorbance is measured to create a standard curve
    - Unknown sample is exposed to same conditions and the concentration is determine based on the standard curve
- Very accurate when only one type of protein is present in the solution
- Limited by the presence of detergent in the sample or by excessive buffer.