Characterization of protein (Pre-formulation study)

Lecture Three

Monday: 6 / 3 / 2023

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The proteins used for therapeutic applications should have:

Well-defined structure, pharmacology, and mechanism of action.

In addition, protein behavior in solution

and interaction with each other (intermolecular) needs to be well defined.

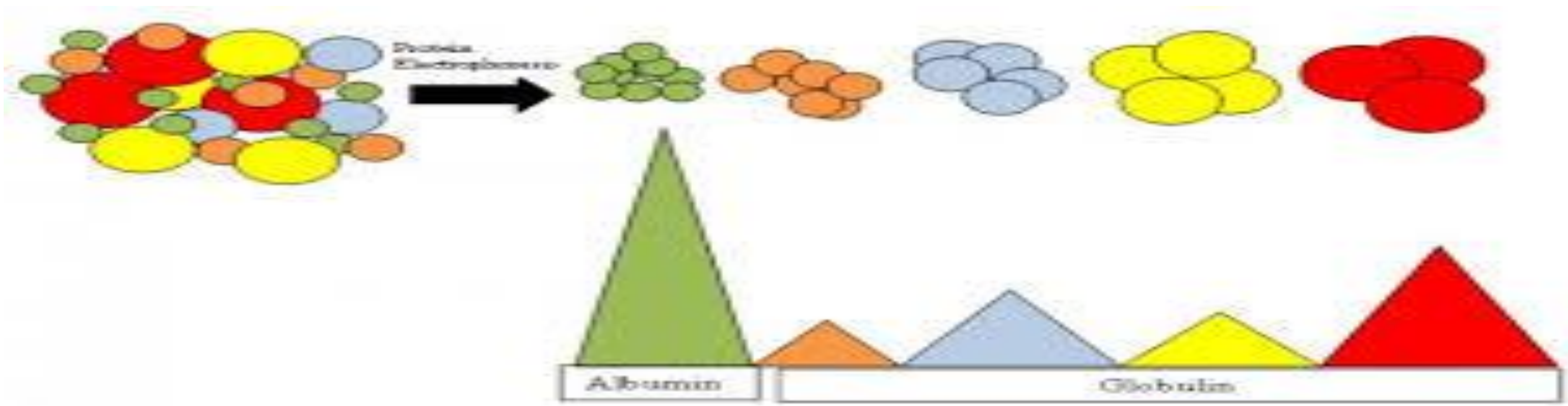
Protein characterization or studying of protein considerations before formulation, plays a critical role in determining the safety and efficacy of biological products and selection of suitable materials and methods for product development.

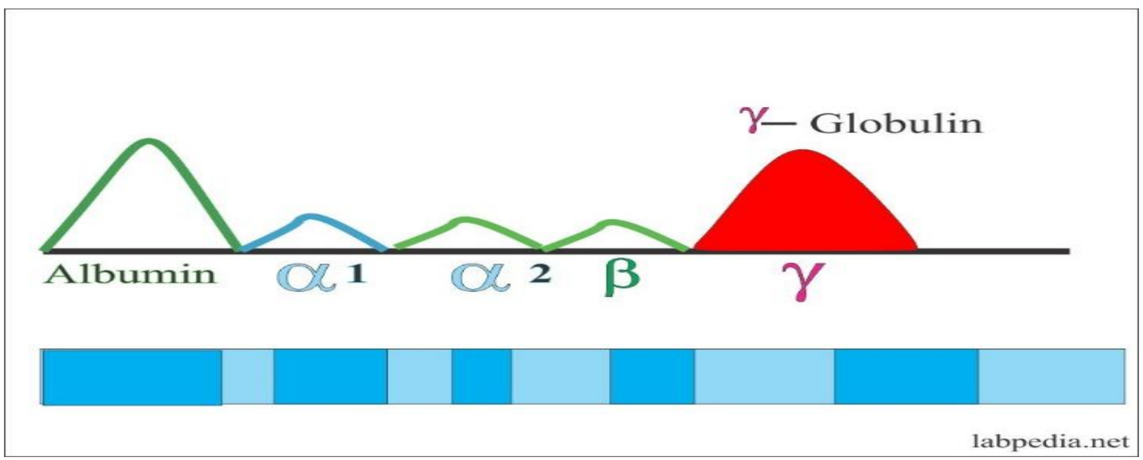
The most important considerations are:

1) Solubility and hydrophobicity
2) Stability
3) Antigenicity and immunogenicity

These considerations can be monitored using different methods like:

1) Electrophoresis to determine protein shape and size in solution (depending on **electrical** properties).
2) Dynamic light scattering (**DLS**) to determine homogeneity of protein solution and crystallization (**Δ size**).
3) Fluorescence spectroscopy to determine protein **conformation** in solution, effects of formulation components and stability storage on protein conformation.





Normal serum protein electrophoresis

Solubility

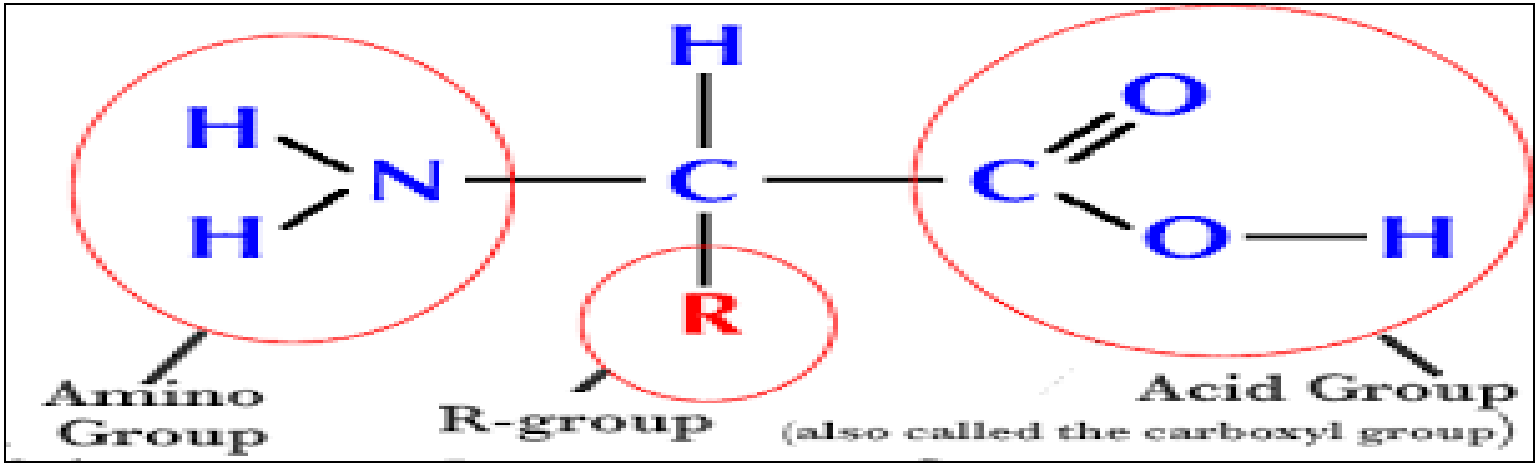
- Under physiological conditions??, solubility of proteins varies enormously from

(**the very soluble to the virtually insoluble**).

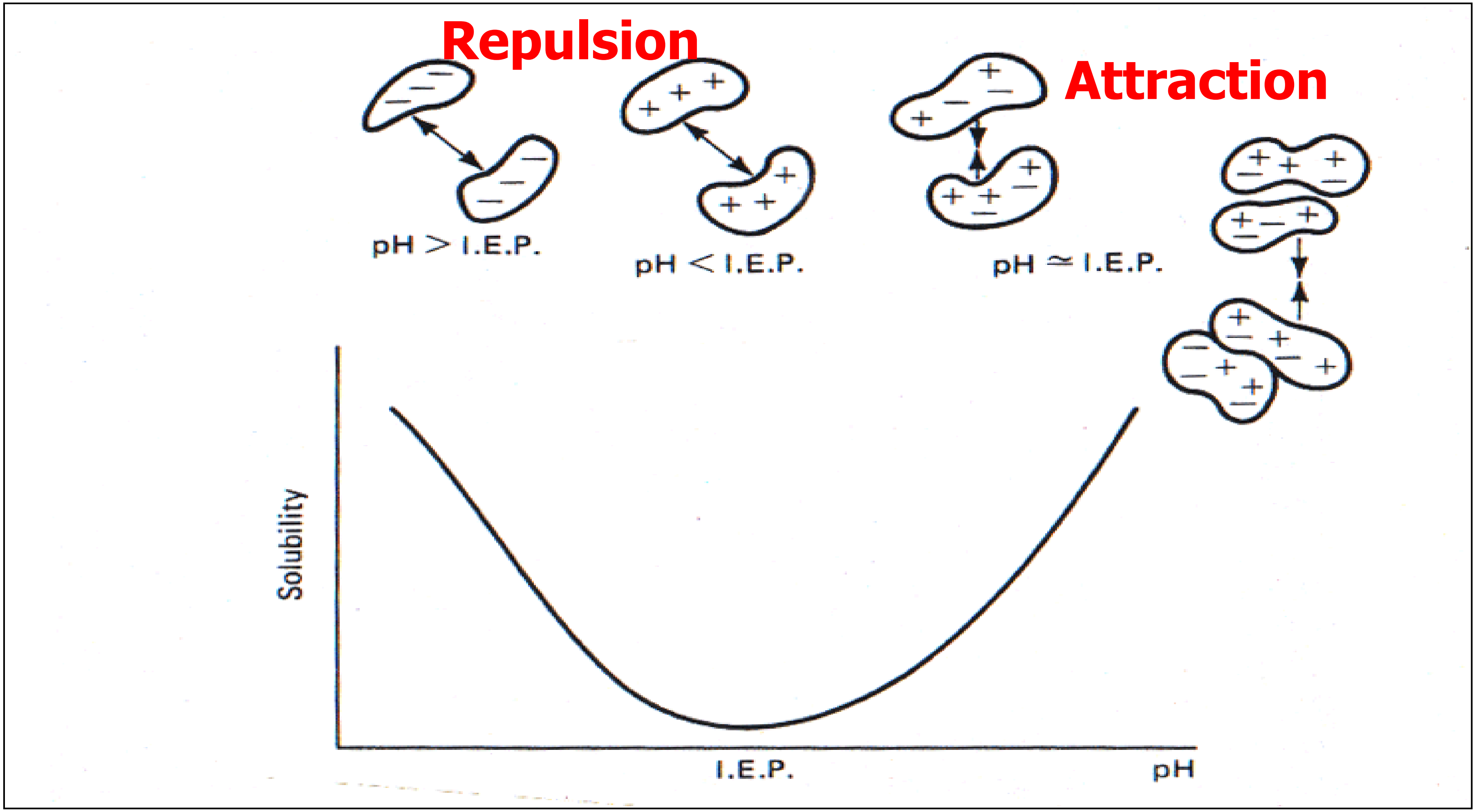
- Water solubility requires interactions, such as hydrogen bonding and electrostatic interactions, of protein surface with the aqueous medium.

- The hydrophilic interactions, which are stronger and predominant in aqueous conditions, are enhanced by the ionization of functional groups on proteins such as amines and carboxylates.
- Ionization of these functional groups is pH dependent.
- **Thus, the solubility of proteins and peptides is dependent on pH of the solution**.

- The overall charge on a protein can be either positive or negative, depending on the ionization status of all of its functional groups.
- **A protein is usually positively charged at a low pH and negatively charged at a high pH.**



- Protein solubility increases as the pH of the solution moves away from the isoelectric point (IEP), which is the pH at which the molecule is ionized but has a net zero charge and does not migrate in an electric field (determined by gel electrophoresis).


Repulsion
Attraction
pH > I.E.P.
pH < I.E.P.
pH ≃ I.E.P.
Solubility
I.E.P.
pH

- The presence of both positive and negative charges on the protein at its IEP leads to *greater tendency for* **self-association**.

- As the net charge on the protein changes in any one direction (positive or negative) with a change in solution pH, the affinity of the protein for the aqueous environment increases and the protein molecules also exert a greater electrostatic repulsion among each other, thus preventing them from self-associating.
- Extremes of pH → Protein unfolding

- The phase behavior of protein solutions is affected by pH, ionic strength, and temperature.
- Generally, protein solubility initially increases with increasing ionic strength of salts, such as NaCl and KCl, but decreases at higher ionic strength, which is called the salting out effect.
- This phenomenon is used to concentrate dilute solutions of proteins and to separate a mixture of proteins (The added salt can then be removed by dialysis).

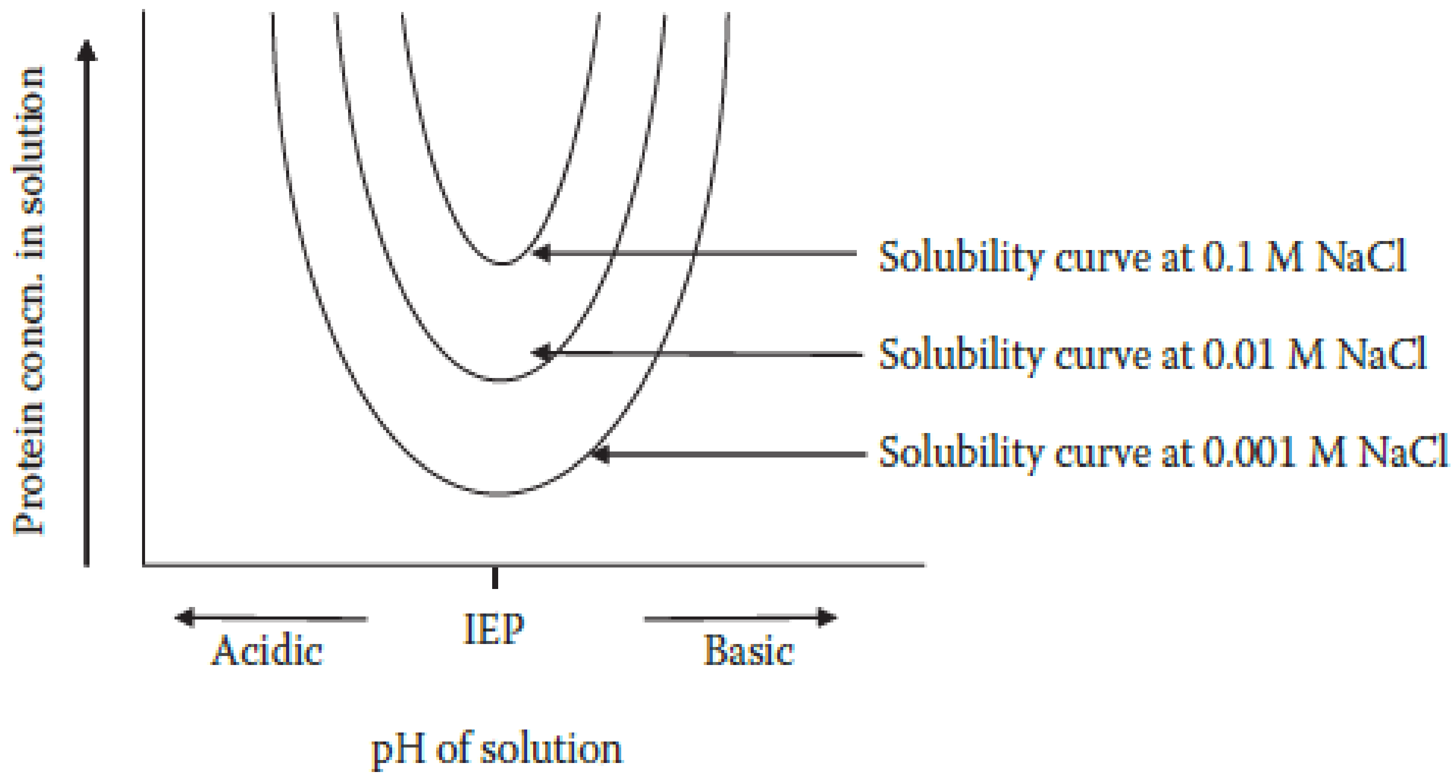
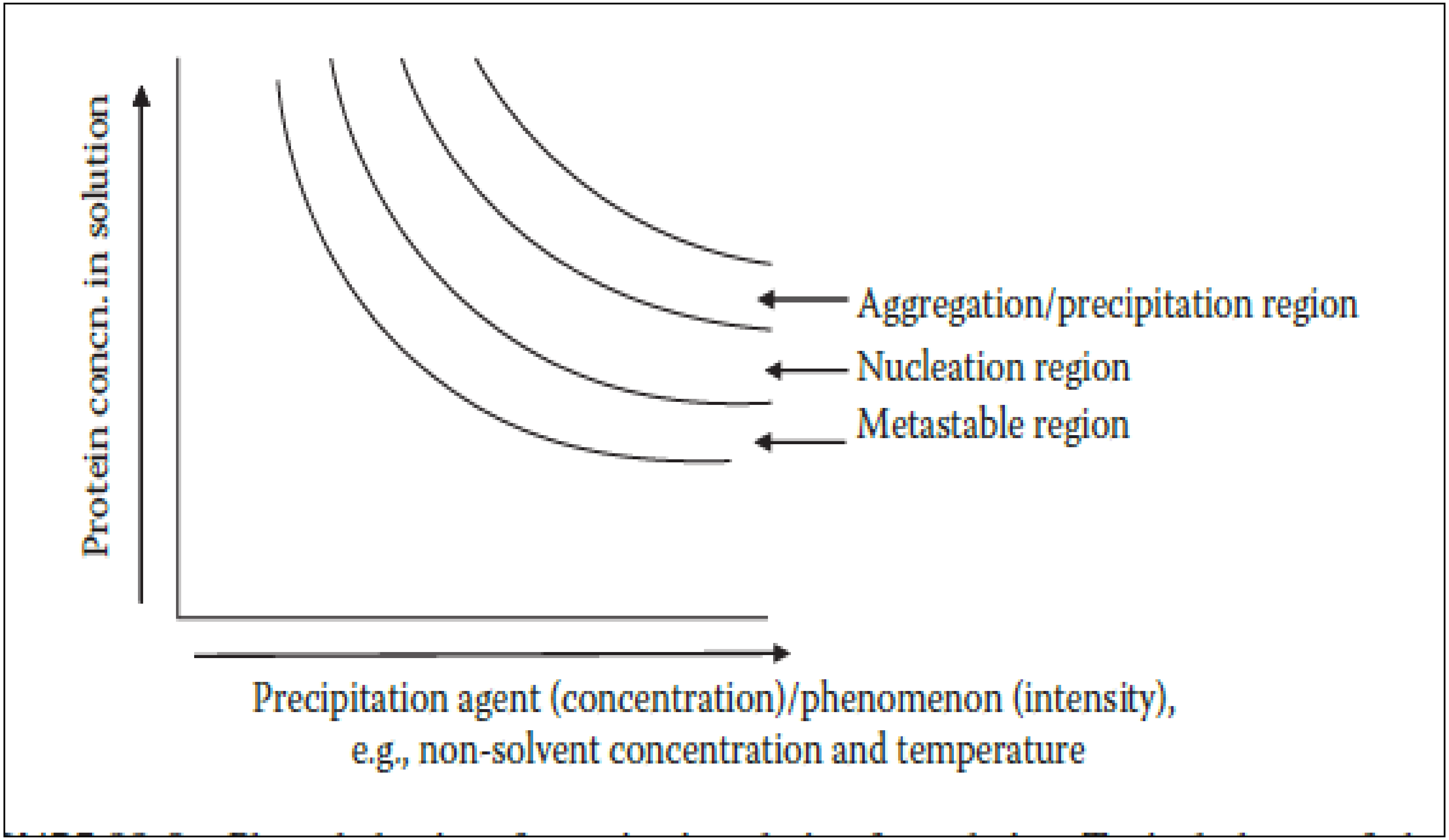


FIGURE 22.7 A typical profile of protein solubility in solution as a function of solution pH and salt concentration.

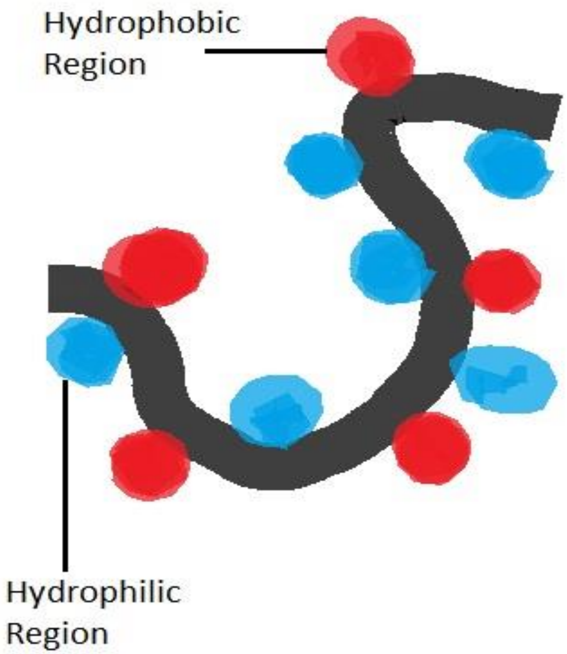
- Organic solvents tend to decrease the solubility of proteins by lowering solvent dielectric constant.
- The presence of other polymers in the solution (cosolutes) also tends to reduce protein solubility by their interactions with solvent molecules, thus tying up the solvent, thus reducing possible protein–solvent interactions. This phenomenon is known as **the volume exclusion effect.**


Protein concn. in solution
Aggregation/precipitation region
Nucleation region
Metastable region
Precipitation agent (concentration)/phenomenon (intensity),
e.g., non-solvent concentration and temperature

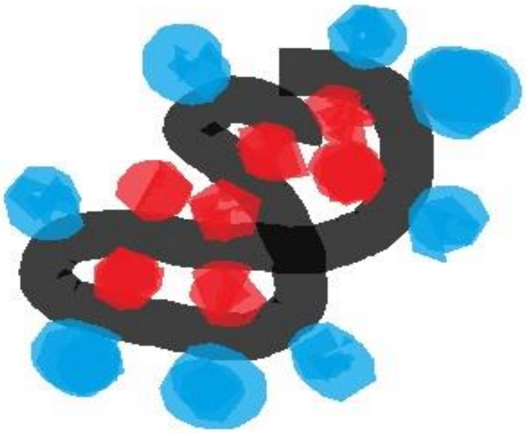
Hydrophobicity

- Different amino acids have different degrees of hydrophobicity.
- If amino acids are spatially arranged in a molecule so that distinct hydrophobic and hydrophilic regions appear on the surface, then the polypeptide or protein will have an amphiphilic nature.

- In an aqueous solution, hydrophobic regions of a polypeptide tend to point away from the hydrophilic aqueous environment to achieve the thermodynamically least energy state of greatest stability.
- In doing so, the hydrophobic surfaces of a protein tend to cluster together on the inside of the protein and form weak van der Waals interactions.

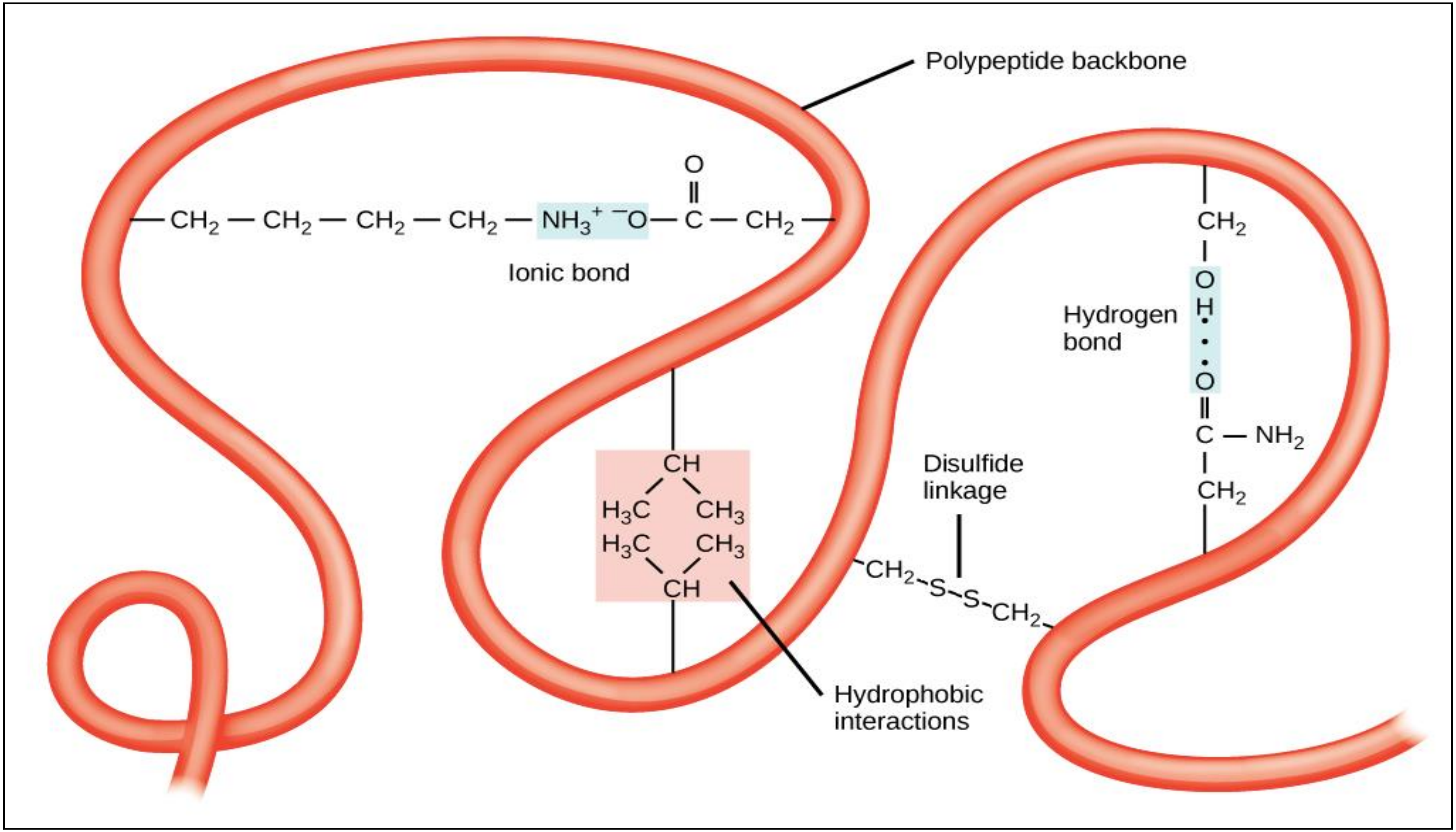


Isolated Protein



Protein in aqueous solution

- Thus, in addition to the stabilizing electrostatic interactions, including van der Waals forces, hydrogen bonds, and ionic interactions, hydrophobic interactions within and among a protein’s polypeptide chains stabilize native protein structure.


Polypeptide backbone
$CH_2 — CH_2 — CH_2 — CH_2 — NH_3^+ \ ^-O — C(=O) — CH_2$
Ionic bond
CH_2
O
H
O
C — NH_2
CH_2
Hydrogen bond
CH
H_3C
CH_3
H_3C
CH_3
CH
Disulfide linkage
CH_2-S-S-CH_2
Hydrophobic interactions