IGBMC
Institut de génétique et de biologie moléculaire et cellulaire

PREPARATION AND CHARACTERIZATION OF BIOLOGICAL SAMPLES FOR STRUCTURAL STUDIES

FROM UNSTABLE PROTEIN TO STABLE COMPLEXES
THE HIV-1 PRE-INTEGRATION COMPLEX, STEROID NUCLEAR RECEPTORS
STRUCTURE, FUNCTION AND DYNAMICS

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Preparation of biological sample

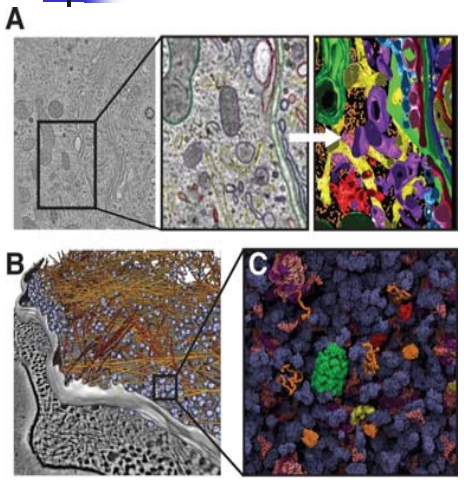
- **Extract a single, pure, homogenous, soluble and functional protein / protein complex from a high complex mixture**

"Cellular crowding"

Intracellular medium: complex mixture

- **Peptides**
  - Amino acids chains (< 6 KD)
- **Proteins**
  - Biological macromolecules (> 6 KD)
  - Nucleic acids
  - Chains of nucleotide triphosphate
- **Polysaccharides**
- **Lipids**
- **Small molecules**
- **.....**

Intracellular complexity



The intracellular environment is extremely crowded. Estimates show that the concentration of biological macromolecules (proteins, nucleic acids, ribonucleoproteins, polysaccharides, etc.) inside cells is in the range of **80–400 mg/mL**. **This corresponds to a volume occupancy of 5%–40% and creates a crowded medium, with considerably restricted amounts of free water.** Such natural intracellular media, being filled with billions of protein molecules and a myriad of DNA, RNA, and polysaccharide molecules are known as "crowded" rather than "concentrated" environments, as, in general, **no individual macromolecular species may be present at high concentration.**

Francois-Xavier Theillet; Andres Binolfi; Tamara Frembgen-Kesner; Karan Hingorani; Mohona Sarkar; Ciara Kyne; Conggang Li; Peter B. Crowley; Lila Gierasch; Gary J. Pielak; Adrian H. Elcock; Anne Gershenson; Philipp Selenko; *Chem. Rev.* **2014,** 114, 6661-6714

Irina M. Kuznetsova, Konstantin K. Turoverov and Vladimir N. Uversky, What Macromolecular Crowding Can Do to a Protein, Int. J. Mol. Sci. 2014, 15(12), 23090-23140

Macromolecular crowding

Obviously, the average spacing between macromolecules in such crowded milieu can be much smaller than the size of the macromolecules themselves. Furthermore, the volume occupied by solutes is unavailable to other molecules because two molecules cannot be in the same place at the same time. As a result, any reactions that depend on available volume can be affected by macromolecular crowding effects.

The thermodynamic consequences of the unavailable volume are called excluded volume effects

Irina M. Kuznetsova, Konstantin K. Turoverov and Vladimir N. Uversky, What Macromolecular Crowding Can Do to a Protein, Int. J. Mol. Sci. 2014, 15(12), 23090-23140

Sample preparation

- **Extraction**
- **Purification**

- **Solubility**
- **Mono-dispersity**
- **Stability**

- **Functional protein**

Solubility, Aggregation, Stability, Function

- Solubility
  - Protein dissolved in aqueous solvent
- Aggregation
  - Protein multimerization
- Stability
  - Folded vs. unfolded state
- Function
  - Biological function of the protein

Protein stability

Forces stabilizing proteins

(1) Based on studies of 138 hydrophobic interaction variants in 11 proteins, burying a $-CH_2-$ group on folding contributes 1.1 ± 0.5 kcal/mol to protein stability.
(2) The burial of non-polar side chains contributes to protein stability in two ways: first, a term that depends on the removal of the side chains from water and, more importantly, the enhanced London dispersion forces that result from the tight packing in the protein interior.
(3) Based on studies of 151 hydrogen bonding variants in 15 proteins, forming a hydrogen bond on folding contributes 1.1 ± 0.8 kcal/mol to protein stability.
(4) The contribution of hydrogen bonds to protein stability is strongly context dependent.
(5) Hydrogen bonds by side chains and peptide groups make similar contributions to protein stability.
(6) Polar group burial can make a favorable contribution to protein stability even if the polar group is not hydrogen bonded.
(7) Hydrophobic interactions and hydrogen bonds both make large contributions to protein stability.

C. Nick Pace, J. Martin Scholtz, Gerald R. Grimsley, Forces stabilizing proteins, FEBS Letters 588 (2014) 2177–2184

Proteins stability

- **Protein stability is the net balance of forces, which determine whether a protein will be in its native folded conformation or a denatured state.**

- **Protein stability refers to the physical (thermodynamic) stability or the chemical stability.**

Chemical stability

- **The chemical stability implies a loss of integrity due to the breaking of chemical bonds.**
  - deamination of asparagine and/or glutamine residues,
  - hydrolysis of the peptide bond of Asp residues at low pH,
  - oxidation of the amino acid Met at high temperature,
  - Elimination of the disulfide bonds,
  - Exchange of the disulfide bonds at neutral pH.
- **Other processes include thiol-catalyzed disulfide interchange and oxidation of cysteine residues.**

The stability of proteins: the thermodynamic stability

- The native state of a protein is achieved when it reaches the state of the lowest free energy. From a thermodynamic point of view, the free energy of a protein depends on enthalpy and entropic contributions.
- The enthalpy contributions include covalent bonds, hydrogen bonds, electrostatic interactions and Van der Waals interactions.
- The entropic contributions involve the hydrophobic effect and conformational entropy

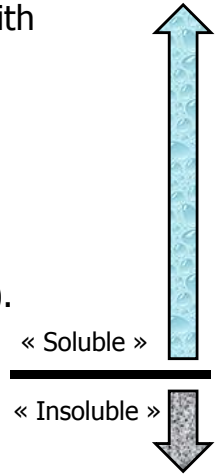
Protein aggregation

Protein aggregation

- Aggregation is a general term that encompasses several types of interactions or characteristics. Aggregates of proteins may arise from several mechanisms and may be classified in numerous ways, including soluble/insoluble, covalent/non-covalent, reversible/irreversible, and native/denatured.
- For protein therapeutics, the presence of aggregates of any type is typically considered to be undesirable because of the concern that the aggregates may lead to an immunogenic reaction (small aggregates) or may cause adverse events on administration (particulates).

Protein aggregation

- **The term aggregate covers a broad spectrum of different types and sizes of associated protein States.**
  - 1. Small reversible non-covalent oligomers with fast kinetics (dimers, trimers, tetramers...).
  - 2. Irreversible non-covalent oligomers.
  - 3. Covalent Oligomers (eg. disulfides).
  - 4. « Large » aggregates (> 10-mer).
    - May be reversible if non-covalent.
  - 5. « very large » aggregates (~50nm à 3µm).
    - May be reversible if non-covalent.
  - 6. Visible particles.

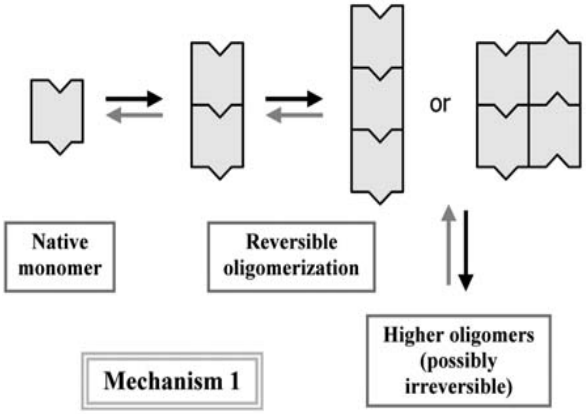


Protein aggregation

- **3 types of protein aggregation.**
  - Natural and productive aggregation as the reaction n(G-actin) →(F-actin)n controlling mobility and shape of cells.
  - Aggregation unwanted in biology including α synuclein, amyloid β, polyglutamine and Prion proteins that play an important role in neuro-degenerative disease as Parkinson's, Alzheimer's, Huntington and the prion-related diseases (mad cow).
  - Aggregation unwanted in vitro important for the conservation of proteins for various applications in research and industry.

Protein aggregation

MECHANISM 1: REVERSIBLE ASSOCIATION OF THE NATIVE MONOMER

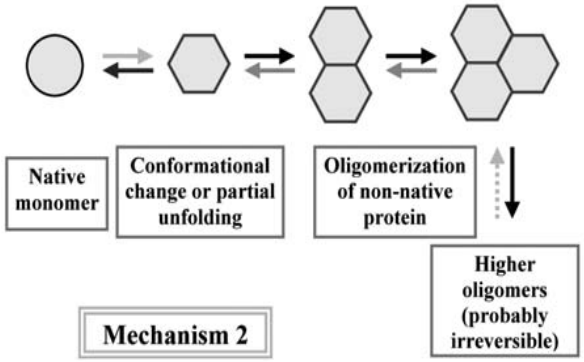


The tendency to reversibly associate (aggregate) is intrinsic to the native form of the protein. The surface of the native protein monomer is self-complementary so it will readily self-associate to form reversible small oligomers. There may be multiple "sticky" or complementary patches on the monomer surface. Those can then produce different types of interfaces, potentially producing multiple conformations for oligomers of the same stoichiometry and different patterns of oligomer growth. As the protein concentration rises and larger and larger oligomers form (driven by the law of mass action), over time these larger aggregates often become irreversible (sometimes through formation of covalent bonds such as disulfide linkages).

John S. Philo and Tsutomu Arakawa, Current Pharmaceutical Biotechnology, 2009, 10, 348-351

Protein aggregation

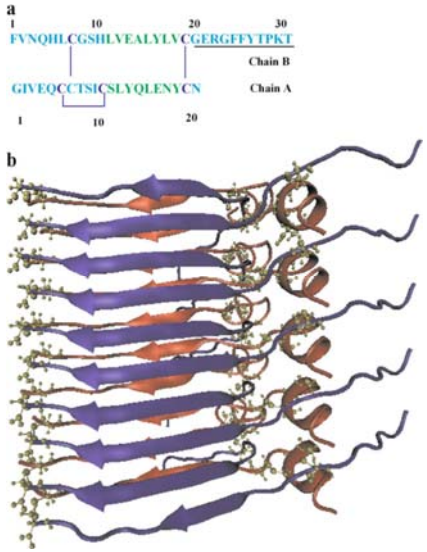
MECHANISM 2: AGGREGATION OF CONFORMATIONALLY-ALTERED MONOMER



John S. Philo and Tsutomu Arakawa, Current Pharmaceutical Biotechnology, 2009, 10, 348-351

In contrast to Mechanism 1, **for Mechanism 2 the native monomer has a very low propensity to reversibly associate. However after it transiently undergoes a conformational change or partial unfolding the resultant altered conformation of monomer associates strongly** (in a manner similar to Mechanism 1). Thus a key difference between Mechanisms 1 and 2 is that in Mechanism 2 the first step is a conformational change to a non-native state, and at any given time the fraction of protein in that aggregation-prone non-native state will usually be quite small. For Mechanism 2 **aggregation will be promoted by stresses such as heat or shear that may trigger the initial conformational change.** A further (and important) consequence is that **aggregation will be inhibited by excipients or conditions that stabilize the native conformation. This aggregation mechanism does appear to be the dominant one for many proteins.** Two therapeutics where this mechanism has been reported are interferon-γ and G-CSF.

Protein aggregation: mechanism 2



Amino acid sequences and structural models of double layer insulin oligomers.

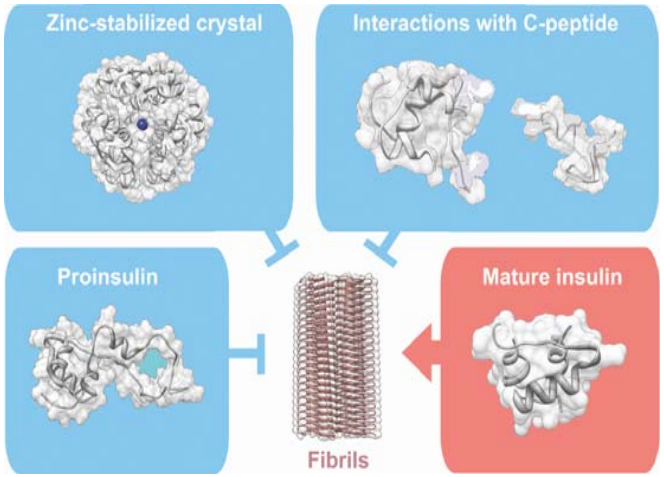
a. Amino acid sequence of insulin. Segments LVEAYLV of chain B and SLYQLENT of chain A are colored green. Disulfide bonds are colored blue. The C-terminal region of chain B (underlined) is not involved in amyloid fibrillation.

b. Single-layered structural models of insulin oligomers (ten-stranded). Two chains are associated via an inter-digitated pair of LYQLENY molecules of chain A and LVEALYL molecules of chain B, which interlock tightly to form the dry steric zipper. Chain A is red and chain B is shown in blue. Disulfide bonds are indicated in yellow

Workalemahu Mikre Berhanu & Artëm E. Masunov, J Mol Model (2012) 18:1129–1142

Protein aggregation: mechanism 2

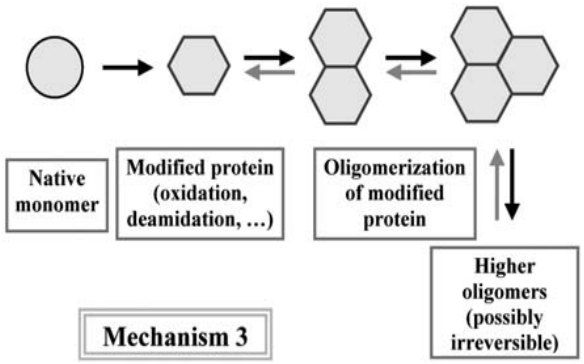
Multiple strategies for the prevention of insulin fibrillation



Biochemical Journal 2012 447, 185-192 - Michael Landreh, Jan Johansson and others.

Protein aggregation

MECHANISM 3: AGGREGATION OF CHEMICALLY MODIFIED PRODUCT



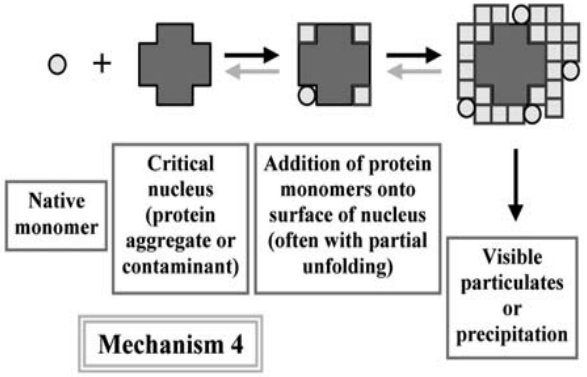
Mechanism 3 is really a variant of mechanism 2 where the **change in protein conformation that precedes aggregation is caused by a difference in covalent structure. Usually this difference is caused by chemical degradation such as oxidation of methionine, deamidation or proteolysis**. Chemical changes may for example create a new sticky patch on the surface, or change the electric charge in a way that reduces electrostatic repulsion between monomers.

In some cases however the chemically different species is not a degradant but rather it is a normal variant within the bulk drug product (for example in glycoproteins there might be a unglycosylated or under glycosylated fraction that is prone to aggregation.

John S. Philo and Tsutomu Arakawa, Current Pharmaceutical Biotechnology, 2009, 10, 348-351

Protein aggregation

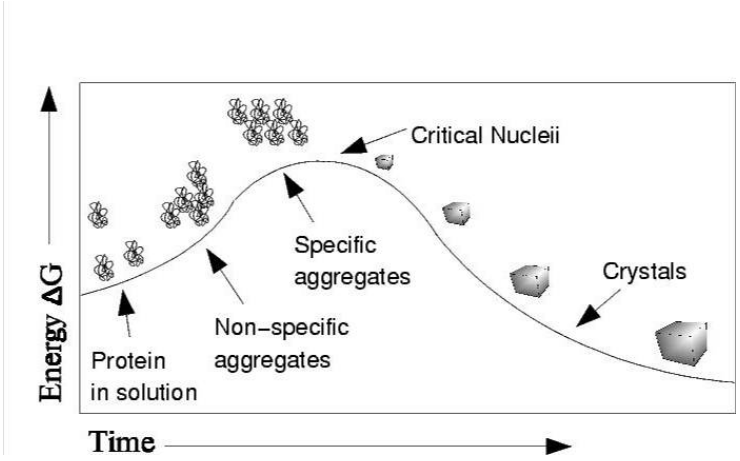
MECHANISM 4: NUCLEATION-CONTROLLED AGGREGATION



Nucleation-controlled aggregation is a common mechanism for formation of visible particulates or precipitates. In this mechanism the native monomer has a low propensity for formation of small and moderately-sized oligomers (the addition of monomers onto these smaller aggregates is not thermodynamically favored). However if an aggregate of sufficient size manages to form, then the growth of this so called "**critical nucleus**" through addition of monomers is strongly favored and the formation of much larger species is rapid. **This type of process is similar to growing large crystals** by adding micro-crystal "seeds" to a saturated solution, and thus the critical nuclei are also sometimes called the "seeds" or "templates" for aggregate growth.

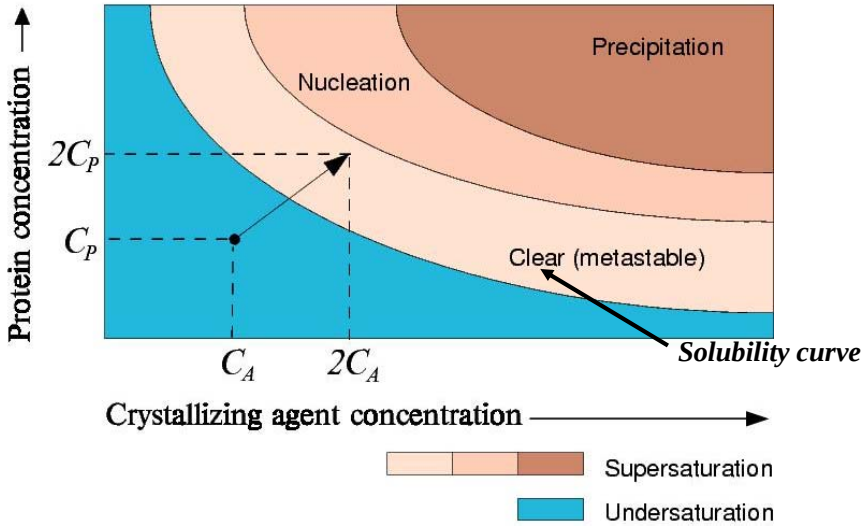
John S. Philo and Tsutomu Arakawa, Current Pharmaceutical Biotechnology, 2009, 10, 348-351

Crystallization

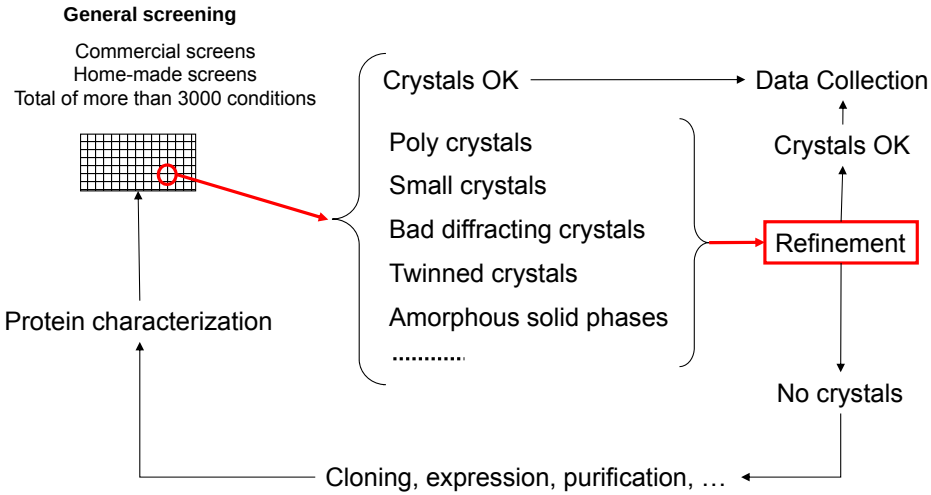


Crystallization

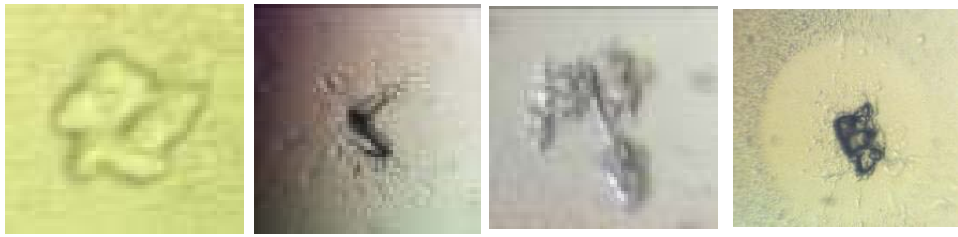
Phase transition



Crystallization assays: general strategy

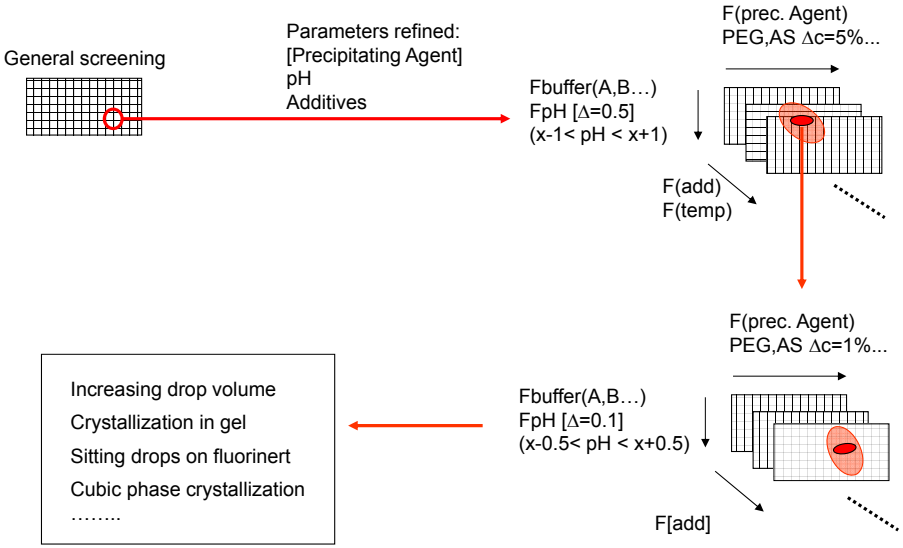


Crystallization assays: amorphous solid phase

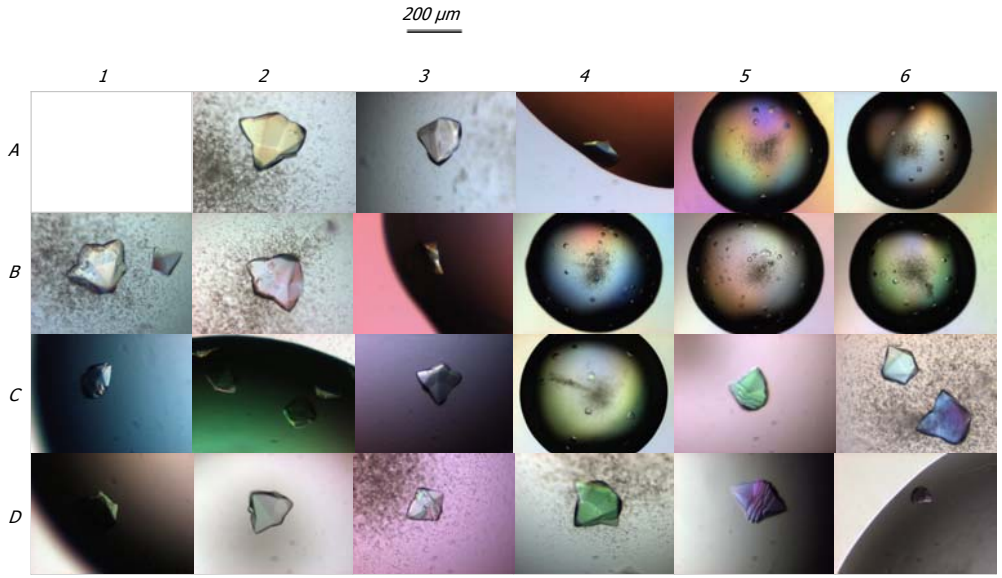


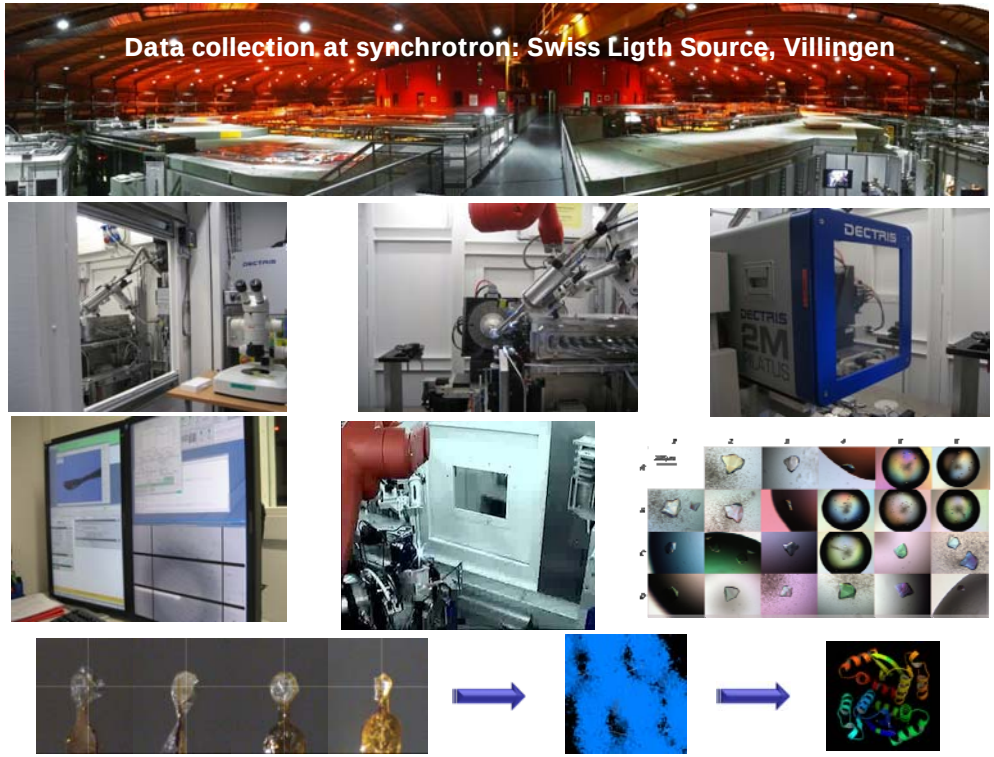
Amorphous solid phase → Crystallization refinement

Crystallization assays: refinement



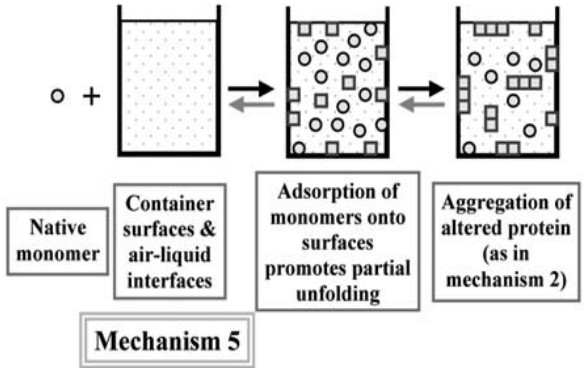
HIV integrase 50 - 212 (F185K), Crystallization





Protein aggregation

MECHANISM 5: SURFACE INDUCED AGGREGATION



This aggregation process starts with binding of the native monomer to a surface. In the case of an air-liquid interface that binding would probably be driven by hydrophobic interactions, but for a container favorable electrostatic interactions might also be involved. After this initial reversible binding event the monomer undergoes a change in conformation (for example to increase the contact area with the surface). Like in Mechanism 2, it is then that conformational altered monomer which aggregates, but in this case that aggregation might occur either on the surface or perhaps after the altered monomer is released back into the solution**. Freeze/thaw damage can also arise from aggregation at the surfaces of ice crystals** or crystals of excipients, and thus can occur through Mechanism 5, but freeze/thaw damage can also involve other mechanisms such as changes in pH.

John S. Philo and Tsutomu Arakawa, Current Pharmaceutical Biotechnology, 2009, 10, 348-351

Protein aggregation

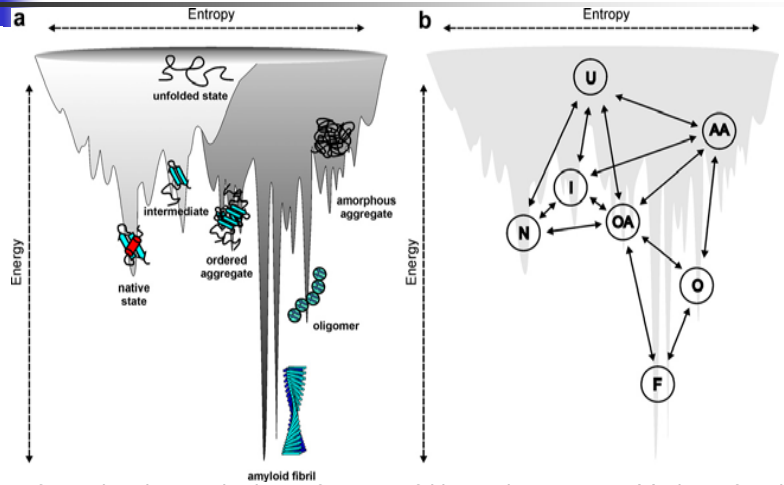
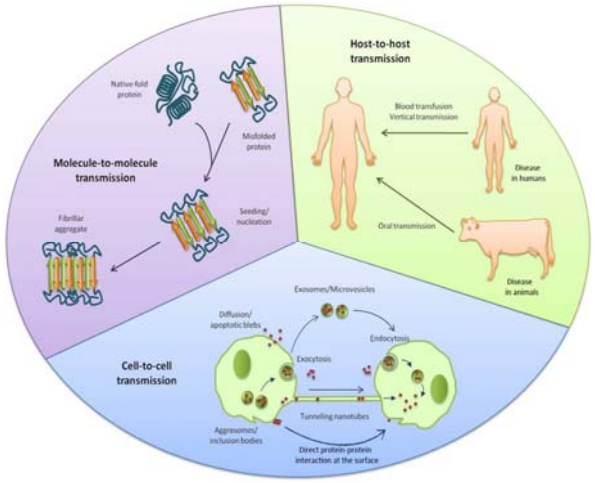


Illustration of a combined energy landscape for protein folding and aggregation. (a) The surface illustrates the roughness of the protein energy landscape, showing the multitude of conformational states available to a polypeptide chain. While rather simple folding funnels (light grey) can describe the conformational search of a single polypeptide chain to a functional monomer, intermolecular protein association dramatically increases ruggedness (dark grey). (b) Proposed pathways linking the conformational states shown in (a) populated on the combined folding and aggregation energy landscape.

T.R. Jahn, S.E. Radford / Archives of Biochemistry and Biophysics 469 (2008) 100–117

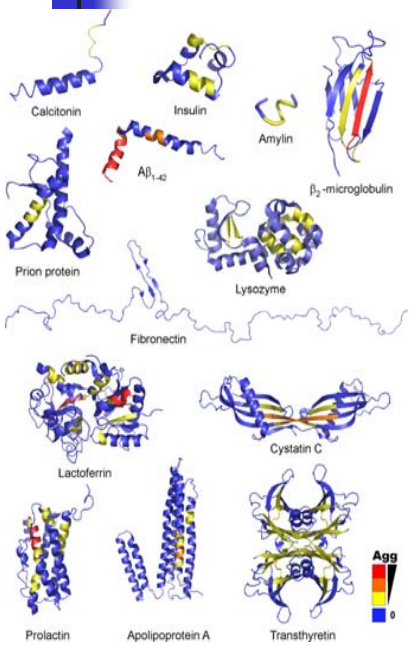
Neuro-degenerative diseases



Transmission of protein misfolding between molecules, cells and individuals. Prion-like transmission of protein misfolding may operate at various levels, including molecule-to-molecule, cell-to-cell and host-to-host. Propagation of the pathological conformational changes and downstream effects to cells, tissues and the entire individual appears to be a universal property of misfolded protein aggregates.

Ines Moreno-Gonzalez, Claudio Soto, Seminars in Cell & Developmental Biology 22 (2011) 482– 487

Protein aggregation



Representative structures of proteins involved in disease-related amyloid fibril formation. The polypeptides are colored according to the aggregation tendency of their amino acid sequences predicted using the algorithm TANGO

Notably, the peptide structures were obtained in the presence of fluoro-alcohols (calcitonin and Ab1–42) or SDS micelles (amylin), and these sequences might be substantially less ordered in the absence of these additives.

T.R. Jahn, S.E. Radford / Archives of Biochemistry and Biophysics 469 (2008) 100–117

Protein aggregation: sequence and gatekeepers

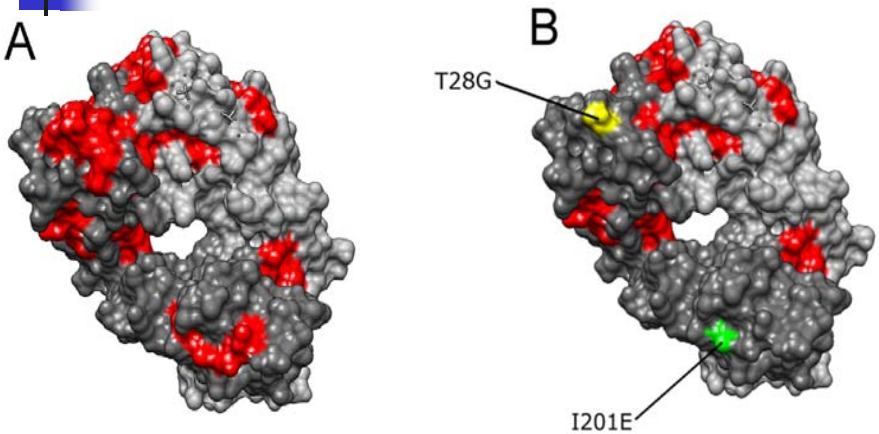
Evolutionary pressure against protein aggregation also results in the placement of amino acids that counteract aggregation at the flanks of protein sequences that are aggregation-prone. These so-called aggregation **gatekeepers** reduce aggregation by opposing nucleation of aggregates.

This disruption is achieved using the repulsive effect of charge (arginine [R], lysine [K], aspartate [D], glutamate [E]), the entropic penalty on aggregate formation (R and K) or incompatibility with β-structure backbone conformation (proline [P]).

The evolutionary enrichment of charged amino acids on the flanks of aggregating regions is coupled to chaperone specificity: studies have shown that chaperones recognize the pattern of charged residues followed by a hydrophobic region. As gatekeeper residues are enriched at the flanks of strongly aggregating hydrophobic sequences, chaperone binding occurs on average more tightly to strongly aggregating than to weakly aggregating sequences

Joke Reumers et al., HUMAN MUTATION, Vol. 30, No. 3, 431–437, 2009

Protein aggregation: sequence and gatekeepers



The crystal structure (space-filling model) of the anti- ErbB2 Fab2C4 (PDB code: 1L7I) is shown. (A). This is a humanized monoclonal antibody fragment that binds to the extracellular domain of the human oncogene product ErbB2 (ErbB2 has been shown to play an important role in the pathogenesis of certain aggressive types of breast cancer). Computationally predicted 'aggregation-prone' regions by AMYLPRED2 are coloured red. Performing only two single amino acid substitutions (T28G and I201E), the AMYLPRED2 output suggests that the antibody has 'lost' two crucial 'aggregation-prone' regions and may, therefore, be more soluble, not forming aggregates (B).

Antonios C. et al, 2013, PLoS ONE 8(1): e54175. doi:10.1371/journal.pone.0054175

Proteins solubility

Proteins solubility

The solubility of a protein in aqueous solution varies from almost completely insoluble to hundreds of milligrams per milliliter. For instance, crambin has been reported to be completely insoluble in water, and serum albumins have solubilities of >500 mg/mL.

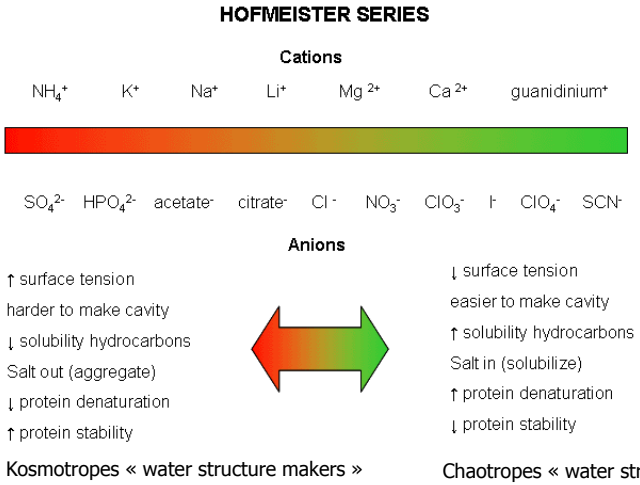
Low protein solubility has also been implicated in a number of human diseases. The P23T mutation in human gD-crystallin shows a markedly decreased solubility and leads to childhood onset of cataracts.

Kramer et al., 2012, Biophysical Journal, 102, 1907-1915

Proteins solubility

- Protein solubility is a thermodynamic parameter defined as the concentration of protein in a saturated solution that is in equilibrium with a solid phase, either crystalline or amorphous, under a given set of conditions
- Solubility can be influenced by a number of extrinsic and intrinsic factors.
  - Extrinsic factors that influence protein solubility include pH, ionic strength, temperature, and the presence of various solvent additives
  - The intrinsic factors that influence protein solubility are defined primarily by the amino acids on the protein surface

Solubility: ionic strength, Hofmeister series



Kosmotropes « water structure makers »

Chaotropes « water structure brakers »

Yanjie Zhang , Paul S Cremer, Current Opinion in Chemical Biology Volume 10, Issue 6 2006 658 - 663

Solubility: ionic strength

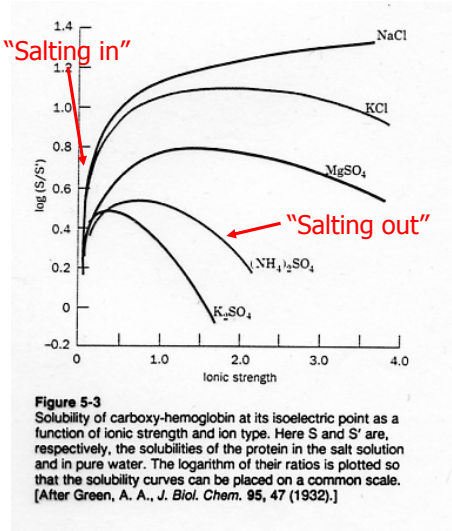


Figure 5-3
Solubility of carboxy-hemoglobin at its isoelectric point as a function of ionic strength and ion type. Here S and S' are, respectively, the solubilities of the protein in the salt solution and in pure water. The logarithm of their ratios is plotted so that the solubility curves can be placed on a common scale. [After Green, A. A., *J. Biol. Chem.* **95**, 47 (1932).]

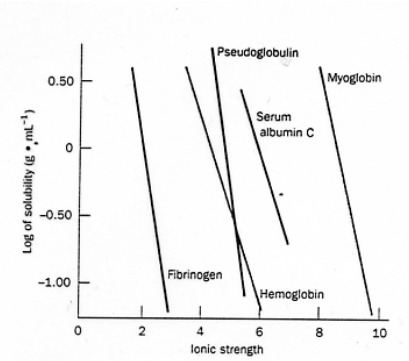


Figure 5-2
Solubilities of several proteins in ammonium sulfate solutions. [After Cohn, E. J. and Edsall, J. T., *Proteins, Amino Acids and Peptides*, p. 602, Academic Press (1943).]

Solubility: ionic strength

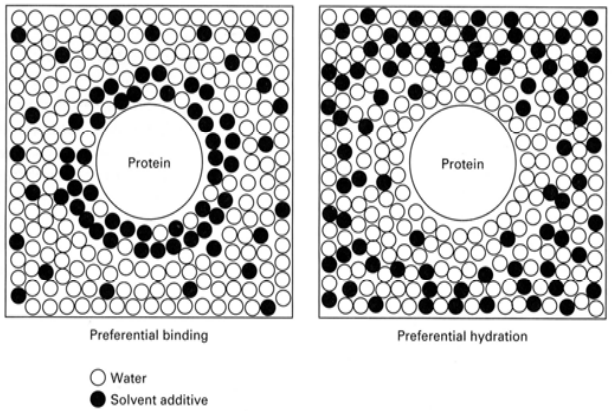
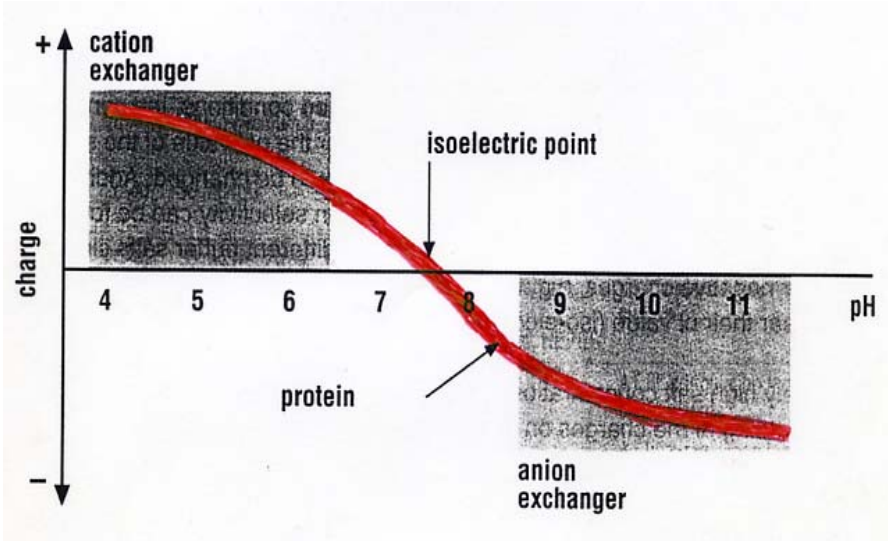


FIGURE 7.14
Schematic illustration of preferential binding and preferential hydration by solvent additives. In preferential binding, the additive occurs in the solvation shell of the protein at a greater local concentration than in the bulk solvent. Preferential hydration results from exclusion of the additive from the surface of the protein. (From S. N. Timasheff and T. Arakawa, in *Protein Structure: A Practical Approach*, T. E. Creighton, ed., pp. 331–345. IRL Press, Oxford, 1989.)

Proteins solubility

Protein net charge in function of pH



Solubility: pH

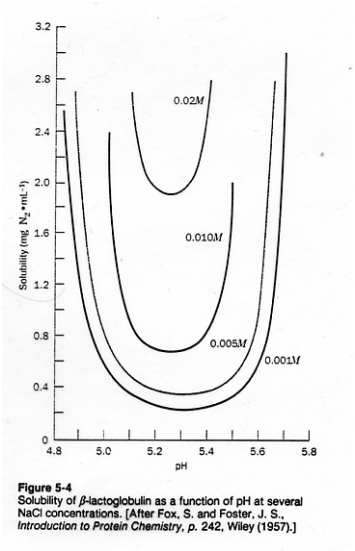


Figure 5-4
Solubility of β-lactoglobulin as a function of pH at several NaCl concentrations. [After Fox, S. and Foster, J. S., *Introduction to Protein Chemistry*, p. 242, Wiley (1957).]

The Isoelectric Points of Several Common Proteins

Protein	Isoelectric pH
Pepsin	<1.0
Ovalbumin (hen)	4.6
Serum albumin (human)	4.9
Tropomyosin	5.1
Insulin (bovine)	5.4
Fibrinogen (human)	5.8
γ-Globulin (human)	6.6
Collagen	6.6
Myoglobin (horse)	7.0
Hemoglobin (human)	7.1
Ribonuclease A (bovine)	7.8
Cytochrome *c* (horse)	10.6
Histone (bovine)	10.8
Lysozyme (hen)	11.0
Salmine (salmon)	12.1

Solubility: hydrophobicity

Capacity of non-polar areas of one or several molecules to come together to minimize the exposure of the hydrophobic area to solvent

TABLEAU 1 — PROPRIÉTÉS DE CERTAINS DÉTERGENTS

Propriétés	Cholate de sodium	*CHAPS*	Octyl glucoside	*Triton X100*	*Lubrol PX*
Poids moléculaire des micelles	1 700	6 150	8 000	90 000	64 000
Concentration micellaire critique % (p/v)	0,36	0,49	0,73	0,02	0,006
"Dialysabilité"	oui	oui	oui	non	non
Présence de charges	oui	non	non	non	non

Protein denaturation

Denaturation is a process in which proteins or nucleic acids lose the tertiary structure and secondary structure which is present in their native state, by application of some external stress or compound such as a strong acid or base, a concentrated inorganic salt, an organic solvent (e.g., alcohol or chloroform), or heat.

If proteins in a living cell are denatured, this results in disruption of cell activity and possibly cell death. Denatured proteins can exhibit a wide range of characteristics, from loss of solubility to communal aggregation.

Protein denaturation

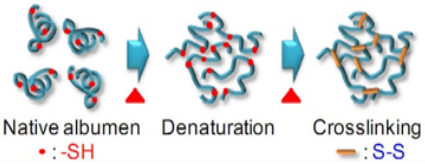
- The denaturing agents are numerous and can be either of physical nature, or be chemical agents.
- Physical agents
  - The increase in temperature causes a thermal agitation of the atoms in the molecule which causes the break of weak interactions such as hydrogen bonds, that stabilize the spatial structure.
  - Change in pH: it leads to a modification of the charges brought by the ionizable groups, and therefore alters the ionic bonds and hydrogen stabilizing the spatial structure.
- Chemical agents
  - Chaotropic agents as urea or guanidine chloride. At high concentrations, these compounds greatly weaken hydrogen bonds (main links of low energies responsible for the maintenance of secondary, tertiary and quaternary protein structures).
  - Thiols reducing agents like 2-mercaptoéthanol or DTT (Dithiothreitol). They allow the reduction (rupture) of the disulfide bonds and can thus contribute to destabilize the tertiary or quaternary protein structure.
  - Bases and acids, by alteration of pH.
  - Detergents, by modification of the interaction with the aqueous solvent.

Protein denaturation

- Heat can be used to disrupt hydrogen bonds and non-polar hydrophobic interactions. This occurs because heat increases the kinetic energy and causes the molecules to vibrate so rapidly and violently that the bonds are disrupted. The proteins in eggs denature and coagulate during cooking. Other foods are cooked to denature the proteins to make it easier for enzymes to digest them. Medical supplies and instruments are sterilized by heating to denature proteins in bacteria and thus destroy the bacteria.

(a)

(b) Protein Thermal Irreversible Denaturation



Protein denaturation

- Alcohol Disrupts Hydrogen Bonding:
- Hydrogen bonding occurs between amide groups in the secondary protein structure. Hydrogen bonding between "side chains" occurs in tertiary protein structure in a variety of amino acid combinations. All of these are disrupted by the addition of another alcohol.
- A 70% alcohol solution is used as a disinfectant on the skin. This concentration of alcohol is able to penetrate the bacterial cell wall and denature the proteins and enzymes inside the cell. A 95% alcohol solution merely coagulates the protein on the outside of the cell wall and prevents any alcohol from entering the cell.

Protein denaturation

- **Heavy Metal Salts:**

- Heavy metal salts act to denature proteins in much the same manner as acids and bases. Heavy metal salts usually contain Hg^{+2}, Pb^{+2}, Ag^{+1} Tl^{+1}, Cd^{+2} and other metals with high atomic weights. Since salts are ionic they disrupt salt bridges in proteins. The reaction of a heavy metal salt with a protein usually leads to an insoluble metal protein salt.

- This reaction is used for its disinfectant properties in external applications. For example $AgNO_3$ is used to prevent gonorrhea infections in the eyes of new born infants. Silver nitrate is also used in the treatment of nose and throat infections, as well as to cauterize wounds.

Protein denaturation

- **Heavy Metal Salts**

- Mercury salts administered as Mercurochrome or Merthiolate have similar properties in preventing infections in wounds.

- This same reaction is used in reverse in cases of acute heavy metal poisoning. In such a situation, a person may have swallowed a significant quantity of a heavy metal salt. As an antidote, a protein such as milk or egg whites may be administered to precipitate the poisonous salt. Then an emetic is given to induce vomiting so that the precipitated metal protein is discharged from the body.

- Heavy metals may also disrupt disulfide bonds because of their high affinity and attraction for sulfur and will also lead to the denaturation of proteins.

Protein denaturation

Thermal denaturation

Ordered molecular systems are usually more stable at low temperatures, where thermal fluctuations are suppressed, but **native proteins tend to be most stable at a temperature, *T**, near room temperature**.

As a consequence, the denaturated state population can be increased either by heating (for $T > T^*$) or by cooling (for $T < T^*$). At sufficiently high or low temperatures, the native protein is thus denatured.

These two ways of disrupting the native protein conformation are known as heat denaturation and cold denaturation, respectively.

The molecular basis of the generic stability maximum of soluble globular proteins and the associated phenomenon of cold denaturation is not fully understood, but hydration effects are generally thought to play a key role.

Monika Davidovic, Carlos Mattea, Johan Qvist, and Bertil Halle, J. AM. CHEM. SOC. 2009, *131*, 1025–1036

Protein denaturation

Overall, protein stability depends on the balance between enthalpy and entropic changes.

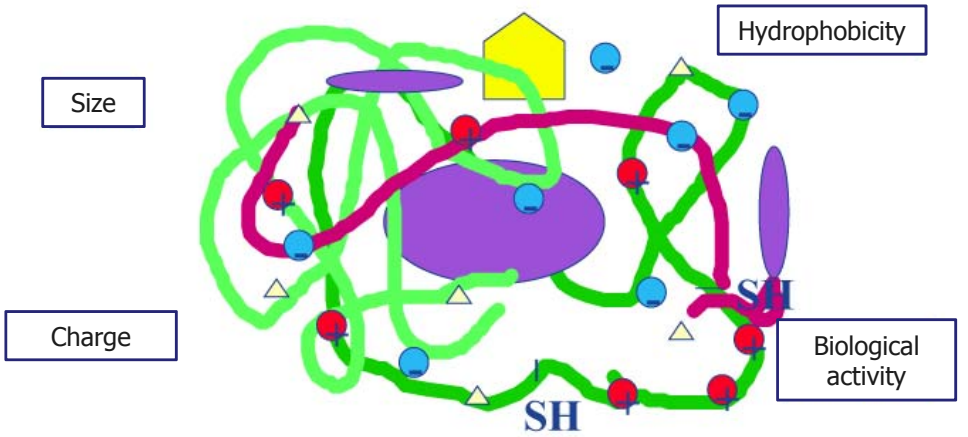
For globular proteins, the free energy of unfolding is commonly found to be positive between about 0 °C and 45 °C. It decreases through zero when the temperature becomes either hotter or colder, with the thermodynamic consequences of both cold and heat denaturation.

Heat denaturation is primarily due to the increased entropic effects of the non-polar residues in the unfolded state. The temperature range for the correct folding of proteins shifts towards lower temperatures if water's hydrogen bonds are weaker and towards higher temperatures if they are stronger.

The hydration of the internal non-polar groups is mainly responsible for cold denaturation as their energy of hydration is greatest when cold. Thus, it is the increased natural structuring of water at lower temperatures that causes cold destabilization of proteins in solution.

Protein purification: how to maintain a protein soluble and functional

Purification



Purification

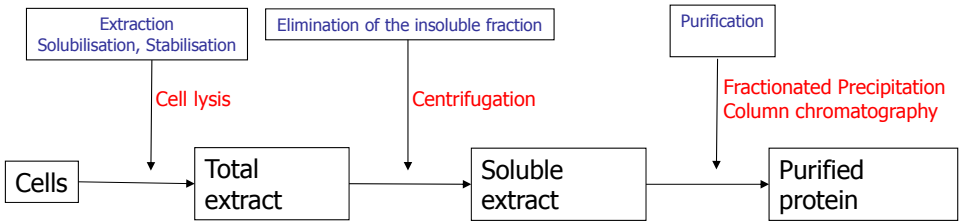
Purification of a protein from a homologous or heterologous organism is to separate the protein of interest from a complex mixture consisting of all cellular components (fatty acids, lipids, carbohydrates, other proteins...)

Cytoplasmic proteins
Secreted proteins
Membrane proteins

Soluble and functional protein

Denatured protein (inclusion bodies)

Purification



Measuring the amount of:
Total protein
Specific protein

Purity analysis

Buffer exchange
Concentration and dilution

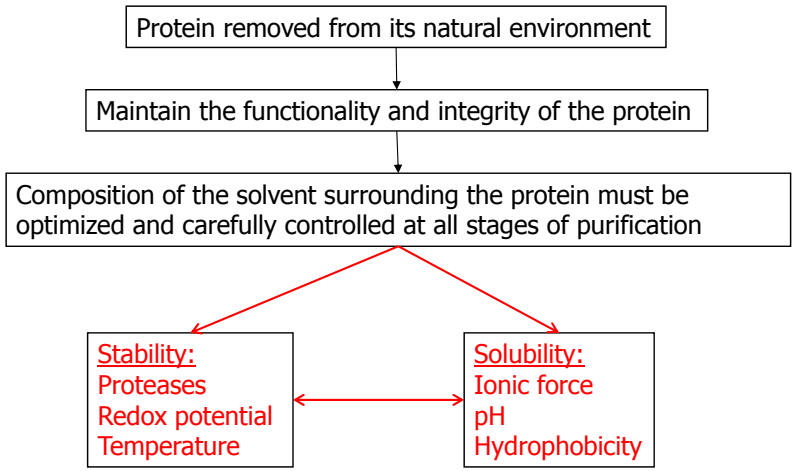
- Adapt the purification to the protein
- Adapt the protein to the purification: fusion proteins for affinity purification (HIS, GST, MBP, biotinilated peptide, STREP, FLAG, ...)

Total extract → Affinity → Tag removal → Affinity → Gel filtration

Cloning and expression

- Protein production in prokaryotic cells
  - E. Coli cells
- Protein production in eukaryotic cells
  - Yeast cells
  - Insect cells
  - Mammalian cells
- Cell free systems

Stabilisation, solubilisation



Stabilisation

- Find the right buffer composition to maintain the integrity of the purified protein
- The release of intra cellular components by grinding releases proteases
- Use of protease inhibitors
- Work at 4°C

Stabilization: REDOX potential

- Potential REDOX (use of reducing agent or oxydo/reducing mixtures)
  - Preventing the formation of inter molecules disulfides bridges
  - Preventing the formation of incorrect intra protein disulfide bridges
  - Maintaining correct disulfide bridge

Solubility

- Ionic force (solubility depending on the concentration and nature of salt)
- Detergents
- Additives (glycerol, fluoro-alcohol, Arg/Glu,…) for protein solubilization
- pH (solubility vs. pH, using buffers)

Solubility: ionic force

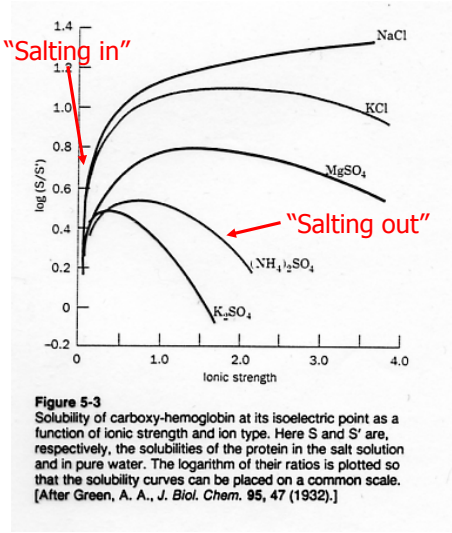


Figure 5-3
Solubility of carboxy-hemoglobin at its isoelectric point as a function of ionic strength and ion type. Here S and S' are, respectively, the solubilities of the protein in the salt solution and in pure water. The logarithm of their ratios is plotted so that the solubility curves can be placed on a common scale. [After Green, A. A., *J. Biol. Chem.* **95**, 47 (1932).]

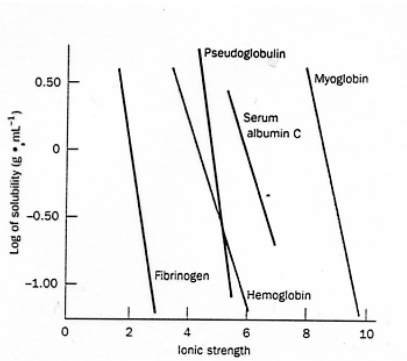


Figure 5-2
Solubilities of several proteins in ammonium sulfate solutions. [After Cohn, E. J. and Edsall, J. T., *Proteins, Amino Acids and Peptides*, p. 602, Academic Press (1943).]

Stabilisation, solubilisation

- Proteins denatured by contact with air - water interface (foam)
- If low amount, a significant fraction may be lost by adsorption to surfaces
- Minimize freezing
- Oxidation of cysteine: addition of reducers (βmercaptoethanol, DTT)
- pH stabilization by buffers
- Stabilization of ionic strength (NaCl, KCl)
- Addition of detergents in the case of aggregation due to hydrophobic interactions
- Proteins denature slowly by chemical or proteolytic degradation: addition of EDTA to remove heavy metals, inhibitors of proteases, purification at 4° C.
- Avoid bacterial contamination: NaN_3

Typical composition of a solution used for cells breaking:

NaCl 100-200 mM, EDTA 0.1 mM, TRIS/HCl pH=7.5, DTT 2 mM, PMSF 0.1 mM