School on Biophysical Approaches to Macromolecules and Cells: Integrated Tools for Life Sciences and Medicine

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Protein Crystallization

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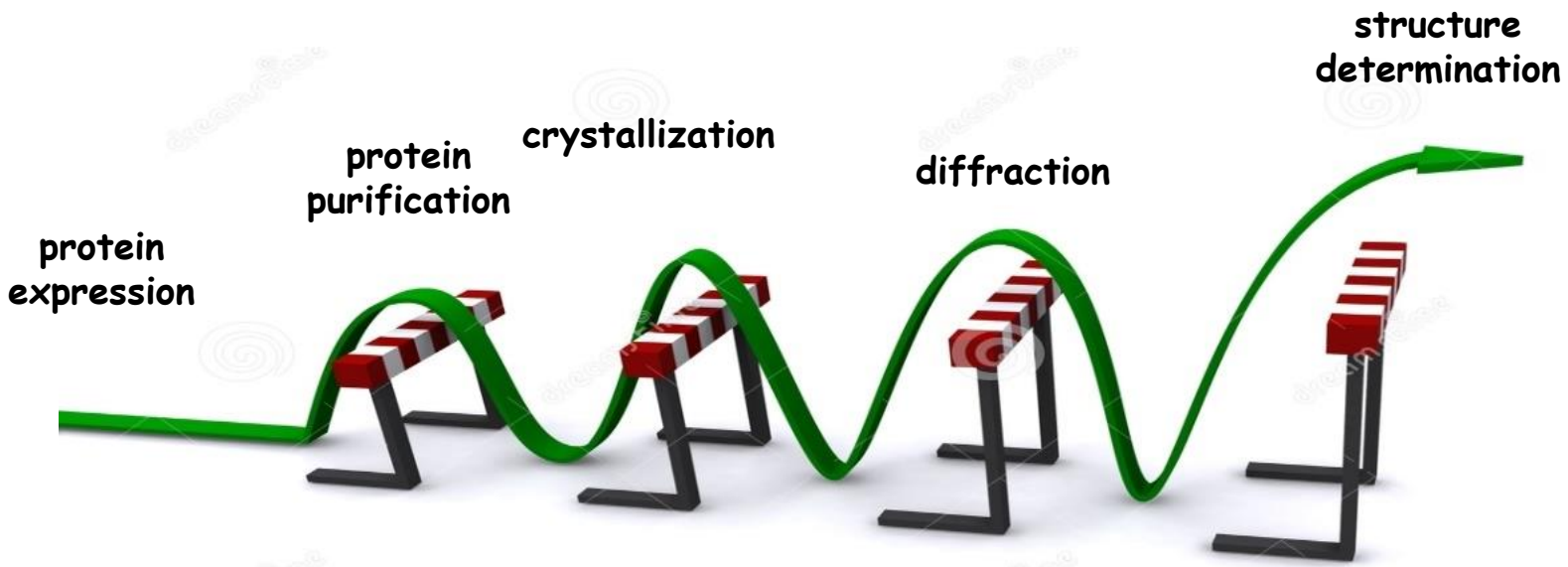
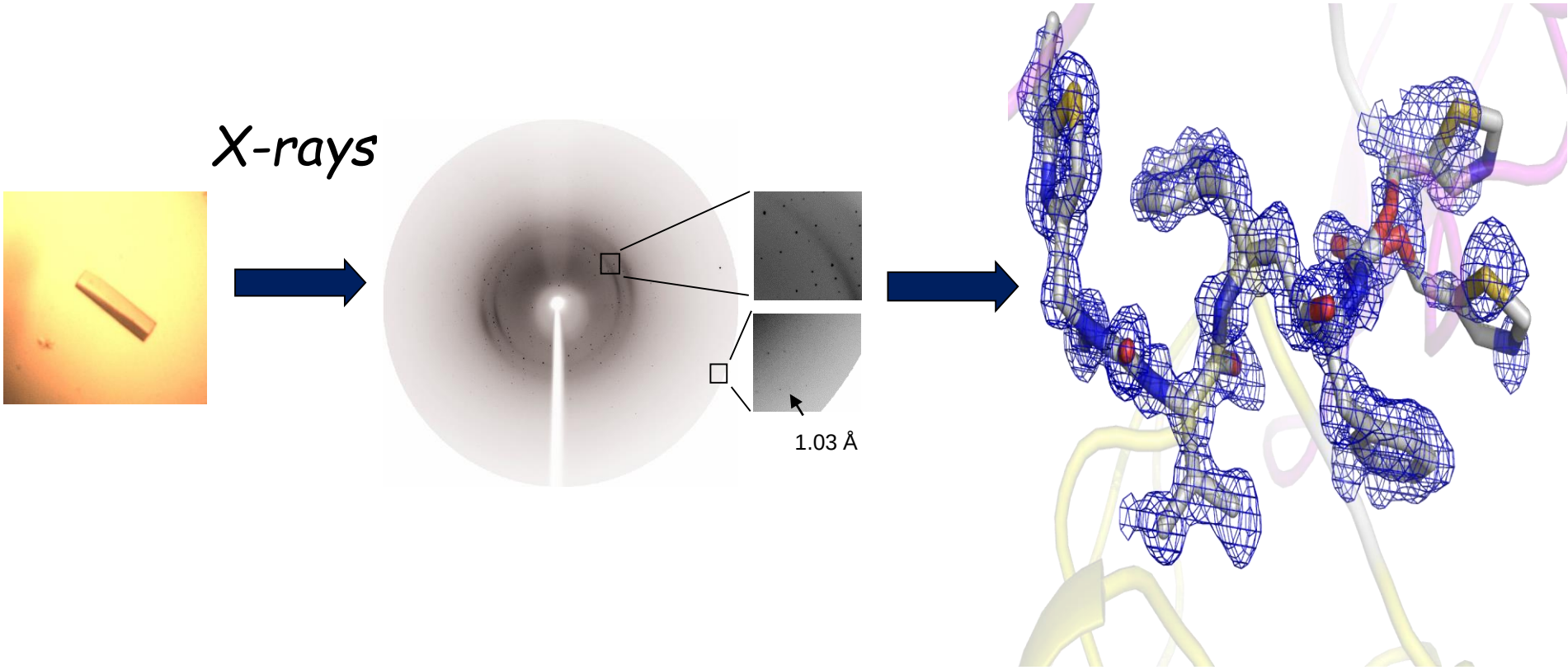
Crystallization: An Hurdle that must be crossed in Biocrystallography



Solving the structure of proteins is pivotal to achieving success in rational drug design and in other biotechnological endeavors. The most powerful method for determining the structure of proteins is X-ray crystallography, which relies on the availability of high-quality crystals. However, obtaining such crystals is a major hurdle.

(Khurshid et al., 2014. Nature Protocols | Vol.9 No.7 | 2014

The Biocrystallography Process



Objectives of Lecture

- Gain knowledge on properties of protein crystals
- Get familiar with the prerequisites for crystallization
- Gain understanding of principles that govern crystal formation and growth
- Understand factors that influence protein crystallization
- Be familiar with various methods of crystallization
- Understand different methods of crystallization screening and optimization

Crystallization and Crystals

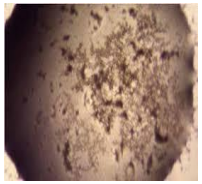
Transition from liquid phase to solid phase

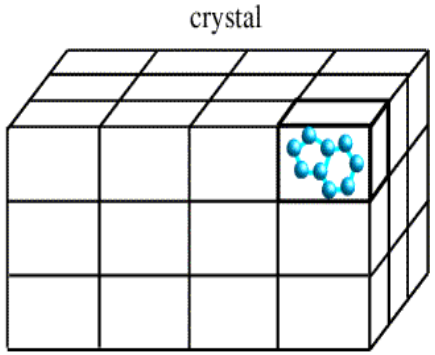
Aqueous protein solution

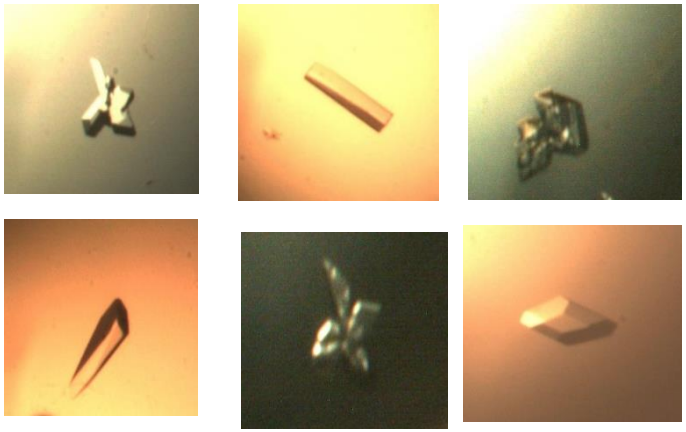
Amorphous solid

or

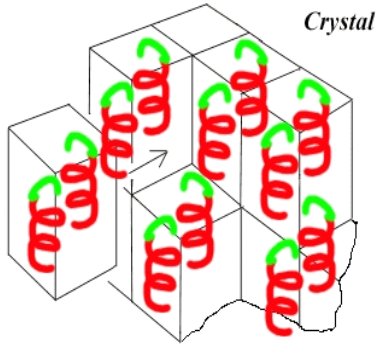
Crystal

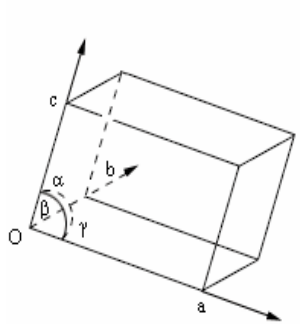






HIV-1 Protease crystals





Parameters of a unit cell-
Edge lengths (a, b, c) and
Internal Angles (α, β, γ)

Crystal Classification

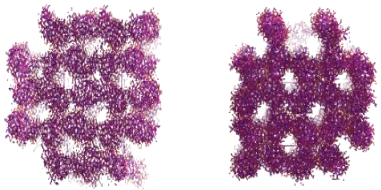
Crystal system	Symmetry	Metric restrictions
Triclinic	None	None **$a \neq b \neq c$; $\alpha \neq \beta \neq \gamma$**
Monoclinic	1 two-fold axis	**$\alpha=\gamma=90$**
Orthorhombic	3 two-fold axes	**$\alpha=\beta=\gamma=90$**
Tetragonal	4 two-fold axes	**$a=b$** **$\alpha=\beta=\gamma=90$**
Trigonal	1 three-fold axis	**$a=b=c$** **$\alpha=\beta=\gamma$** (or Hexagonal)
Hexagonal	6 two-fold axes	**$a=b$** **$\alpha=\beta=90$ $\gamma=120$**
Cubic	4 three-fold axes	**$a=b=c$** **$\alpha=\beta=\gamma=90$**

Properties of Protein Crystals

Protein crystals……

- have large channels of water molecules (>30%)
- are fragile and can break easily
- have very few interactions within molecules producing low order
- are sensitive to external conditions (variation in pH, temperature, ionic strength)
- Have very small dimensions

Channels in Crystal Packing



- Crystal packing shows bonds between atoms and channels occupied by disordered water molecules
- No of channels depends on crystal form
- Channels used to introduce small molecules into crystals or inhibitors/substrates for enzymes by diffusion
- Channels reduce stability of crystal and power of diffraction of a crystal
- Large channels increase the fragility of crystal

Is the Crystal State a Problem?

- < 30% of protein crystal engage in protein-protein interactions
- This makes Protein crystallize in different crystal forms
  - No……..Not a problem!
- Difference in crystal forms approx to 0.1 Å (very small) involving interactions of surface or side chain residues
- Solid state does not influence the overall structure of protein
- Crystal state structure is the same with structure obtained by NMR in solution
- Possibility of catalysis in crystal state for enzymes

Protein Crystallization – Prerequisites

- Protein must be purified (95%) before crystallization
- Homogeneity of protein (uniform conformation) -correctly folded protein)
- Demonstration of biological activity if correctly folded
- Sufficient protein concentration for crystallization trial (5-10 mg/ml)
- Precipitant to use e.g. $(NH_4)_2SO_4$
- Storage temperature must be identified

How to Induce Crystallization

Two Basic Principles!

❖Solubility

Solubility is the maximum concentration of the solute in a given solvent at equilibrium with excess of undissolved compound.

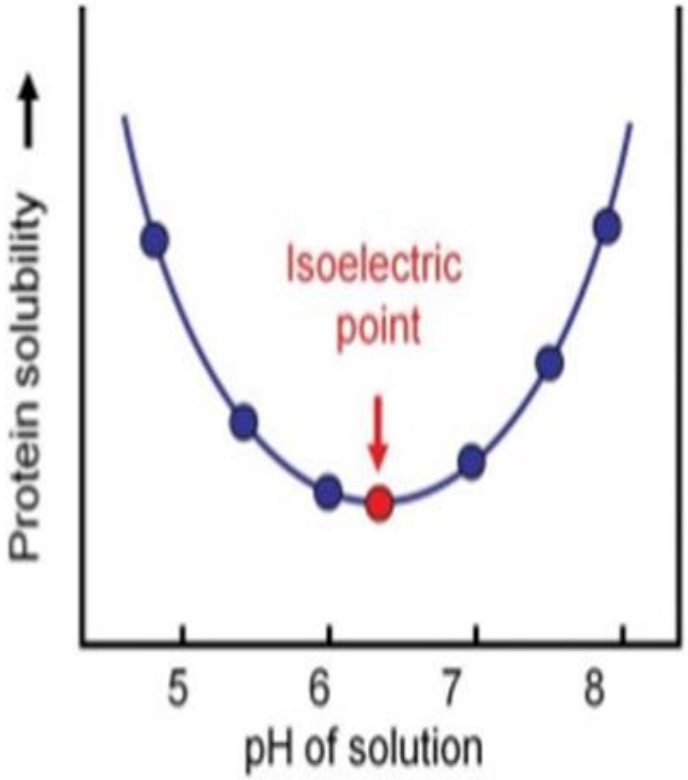
❖Supersaturation

This is a thermodynamically unstable state where more solutes (protein) are dissolved in a solvent than can normally be dissolved.

Two Basic Principles for Crystallization

Solubility and pH

Right choice of buffer in which the protein is both soluble and stable



Two Basic Principles for Crystallization

Solubility and Temperature

- Solubility is dependent on temperature, T
- Entropic effects - Increase in temperature increases the disorder of solvent molecules
- Temperature rise causes vapour to distil away from a drop increasing the degree of supersaturation - shower of crystals
- Decrease in temperature results in vapour condensing on the drop diluting it and increasing the volume

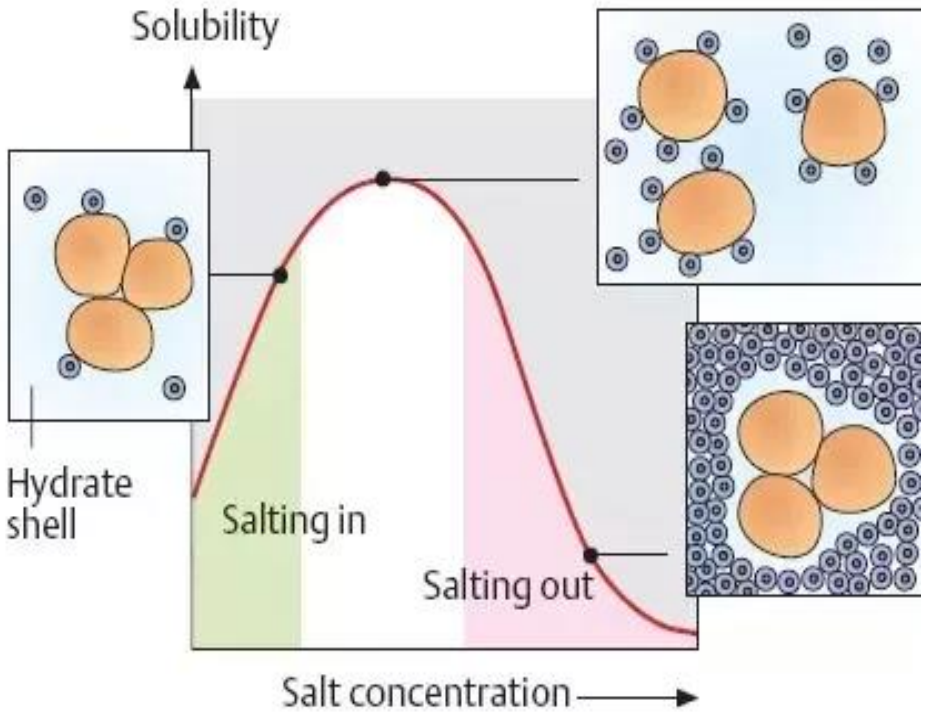
Two Basic Principles for Crystallization

Solubility and Salts

Protein solubility varies with salt concentration in the solution

Salting-in – increasing solubility at low salt concentration

Salting-out – protein solubility is decreased at high ionic strength



Quora.com

Two Basic Principles for crystallization

Supersaturation

- A thermodynamically unstable state where more solutes (protein) are dissolved in a solvent than can normally be dissolved.
- In this state, equilibrium is shifted in favour of the formation of intermolecular bonds.
- Hence, supersaturation is the driving force for crystallization

Two Basic Principles for crystallization

Supersaturation

Methods to achieve supersaturation

- Addition of a precipitant
- Removal of water from protein-precipitant solution (Vapour Diffusion)
- Change of pH

Protein Crystallization Phase Diagram

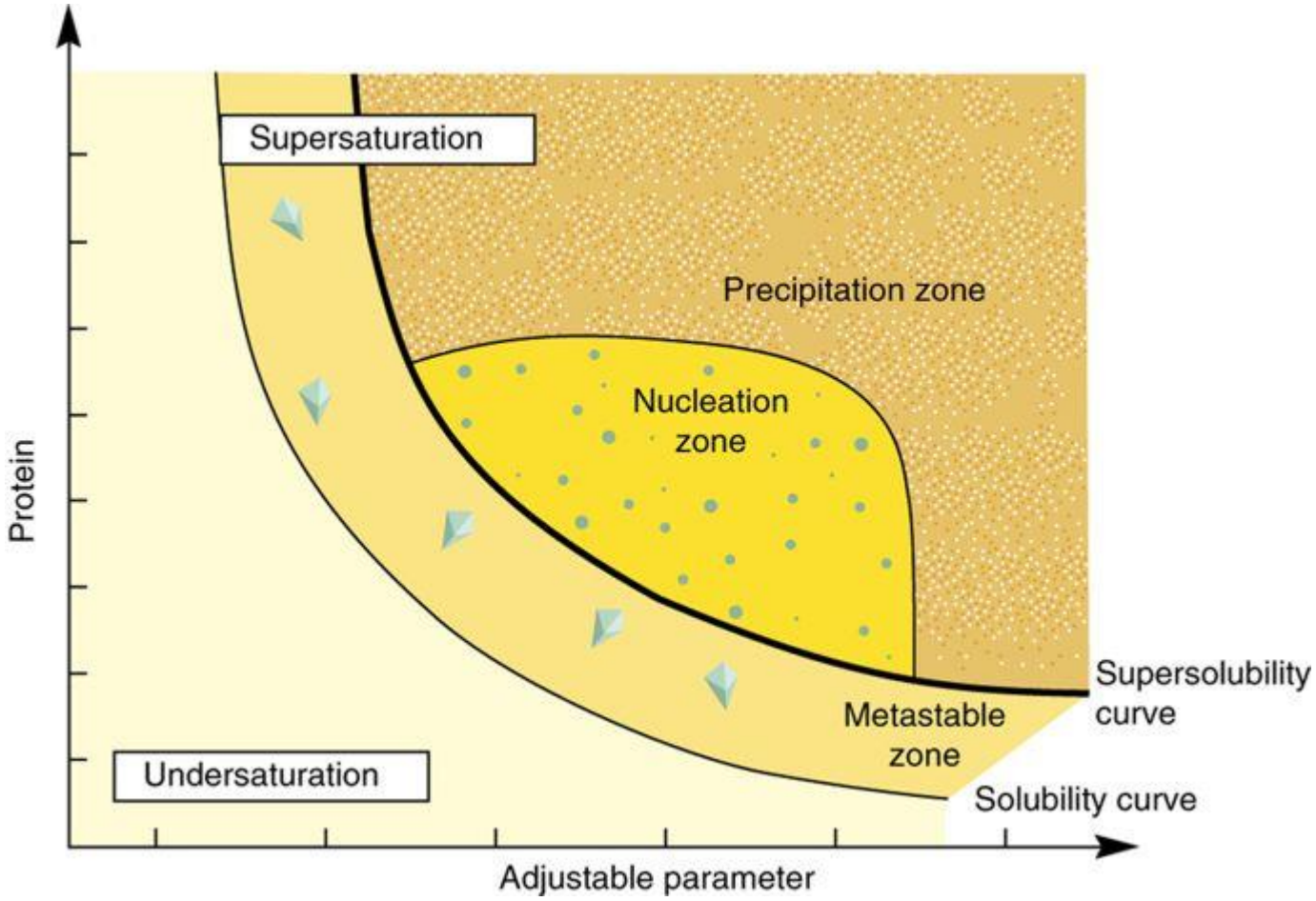
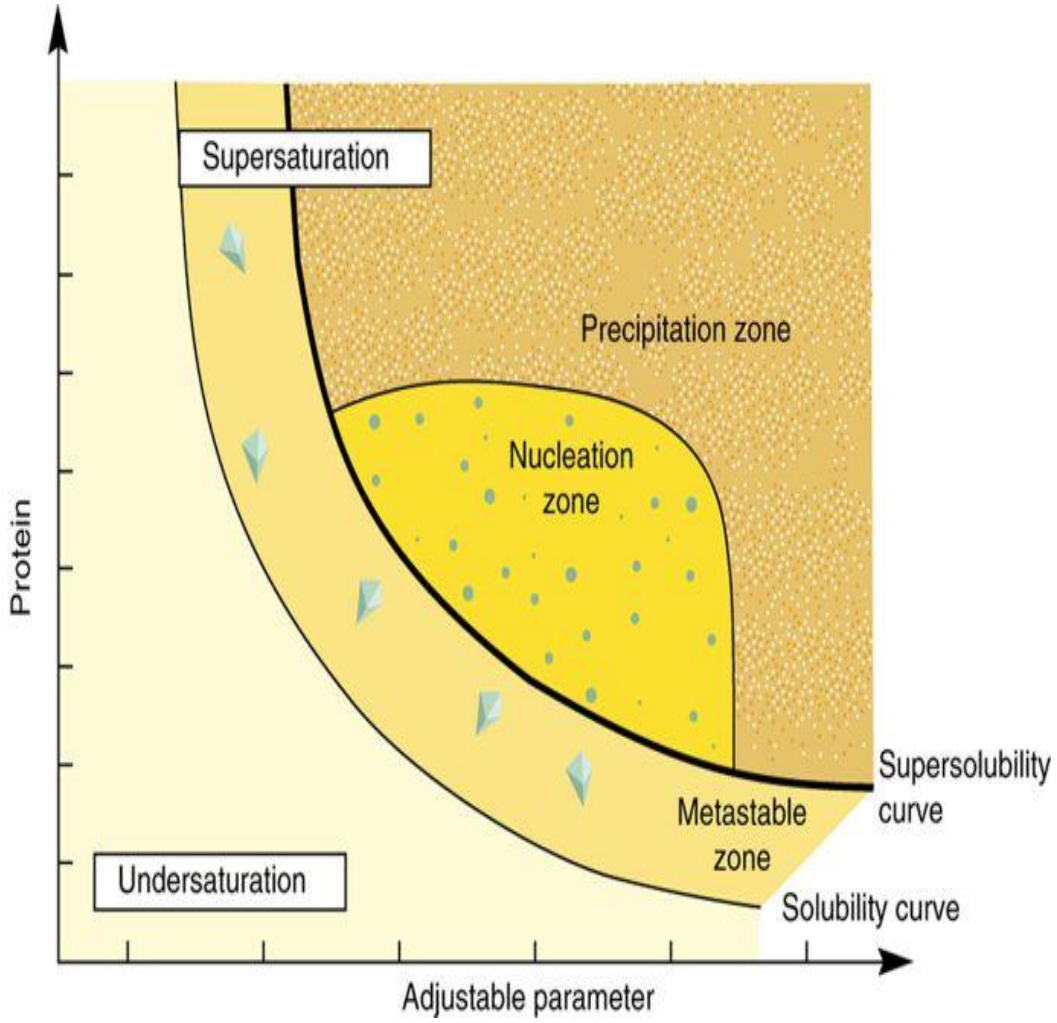


Figure adapted from Chayen, N.E., Turning protein crystallization from an art into a science, Curr. Opin. Struct. Biol., 14, 577–583

Crystallization Process

- Nucleation
- Crystal growth
- Cessation of growth



Factors that influence Crystallization

- Purity of protein
- Concentration of protein with minimum of about 1.5 mg/mL
- Concentration of precipitants (salts, alcohol, straight chain polymers)
- pH of buffer (know the pI of the molecule you are working with)

Factors that influence Crystallization

- Additives
- Temperature
- vibrations

Precipitants

Precipitants modify protein-protein contacts and protein-solvent contacts in such a way to form crystals

- **Salts** - have a role of dehydrating protein (salting out) – Sulphates, Chlorides, Acetates,
- **Organic solvents** - Alcohols (ethanol, butanol, isopropanaol), glycerols, glycoethylene, DMSO
- **Straight chain polymers** - Polyethyleneglycol (PEG)

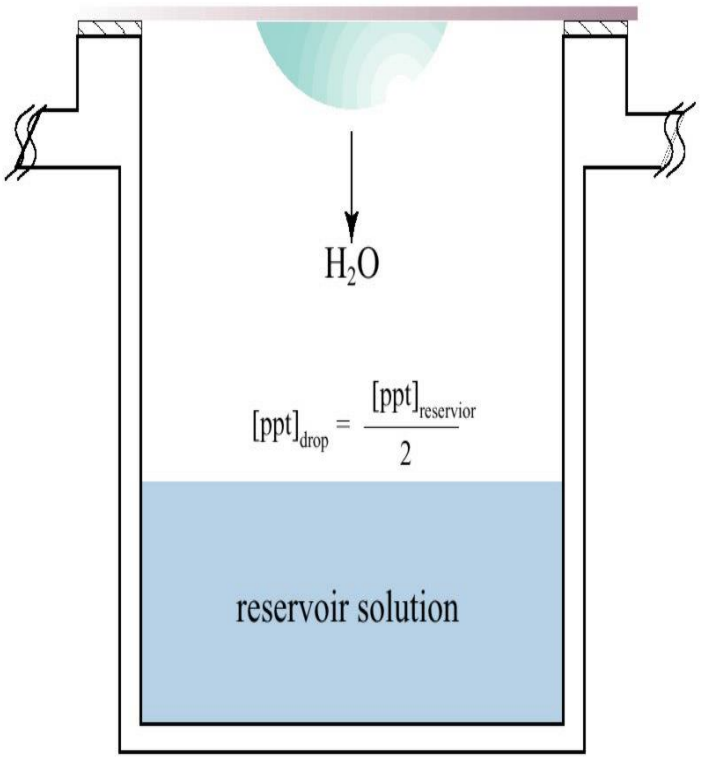
Methods of Crystallization

- **Vapour Diffusion**
  - Hanging drop
  - Sitting drop
- **Batch**
- **Microbatch**
- **Microdialysis**
- **Free Interface Diffusion**

Vapour Diffusion

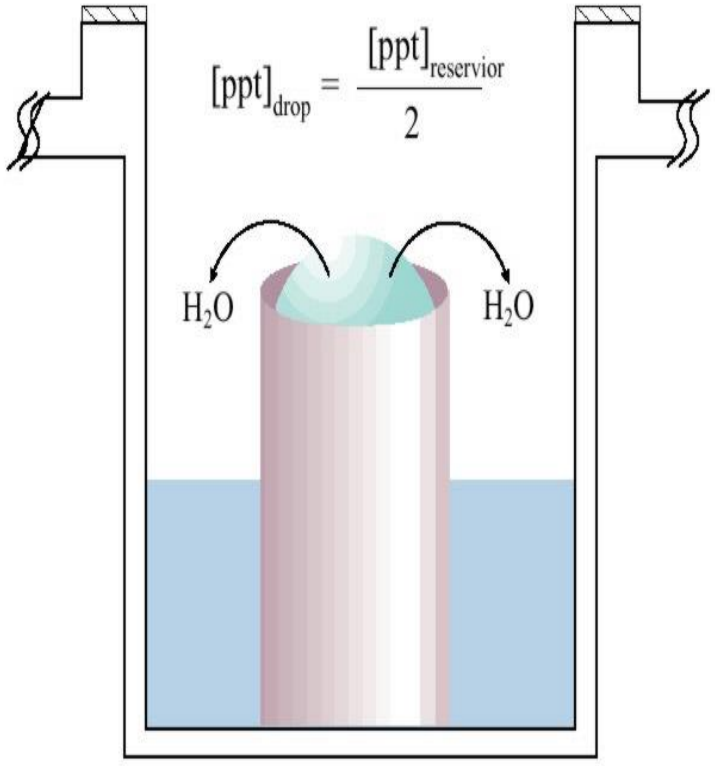
- Very good method for screening large numbers of crystallization conditions
- Evaporation of water from the protein drop is accompanied by net condensation into the reservoir solution equilibrating the concentrations of the two solutions
- Migration of water from the drop results in increased concentration of both the protein and the precipitating agent lowering the solubility of the protein.
- With the right conditions, it induces crystal formation

Vapour Diffusion – by Hanging Drop



- Crystal screen buffer is the reservoir solution (0.5 - 1 mL)
- Drop (on cover slip) is 1/2 protein solution, 1/2 crystal screen buffer (2-4 μL). So, the concentration of precipitant in the drop is 1/2 the concentration in the well.
- Cover slip is inverted over the top of the well and sealed with vacuum grease (airtight)
- The precipitant concentration in the drop will equilibrate with the precipitant concentration in the well via vapor diffusion.

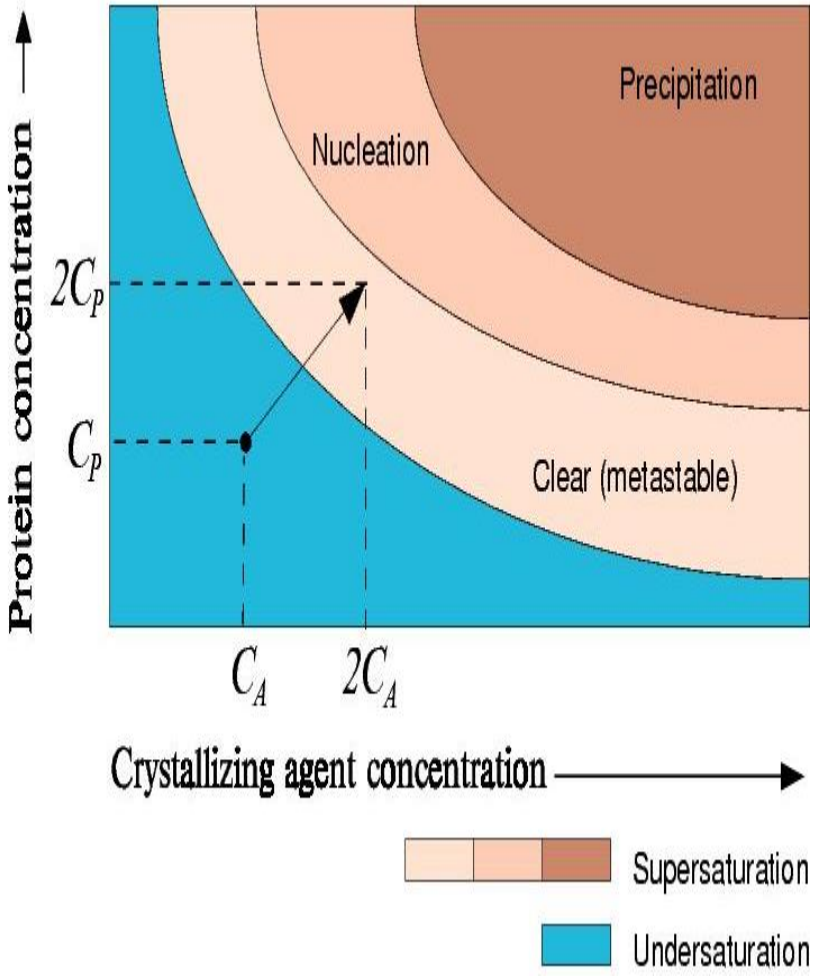
Vapour Diffusion – by Sitting Drop



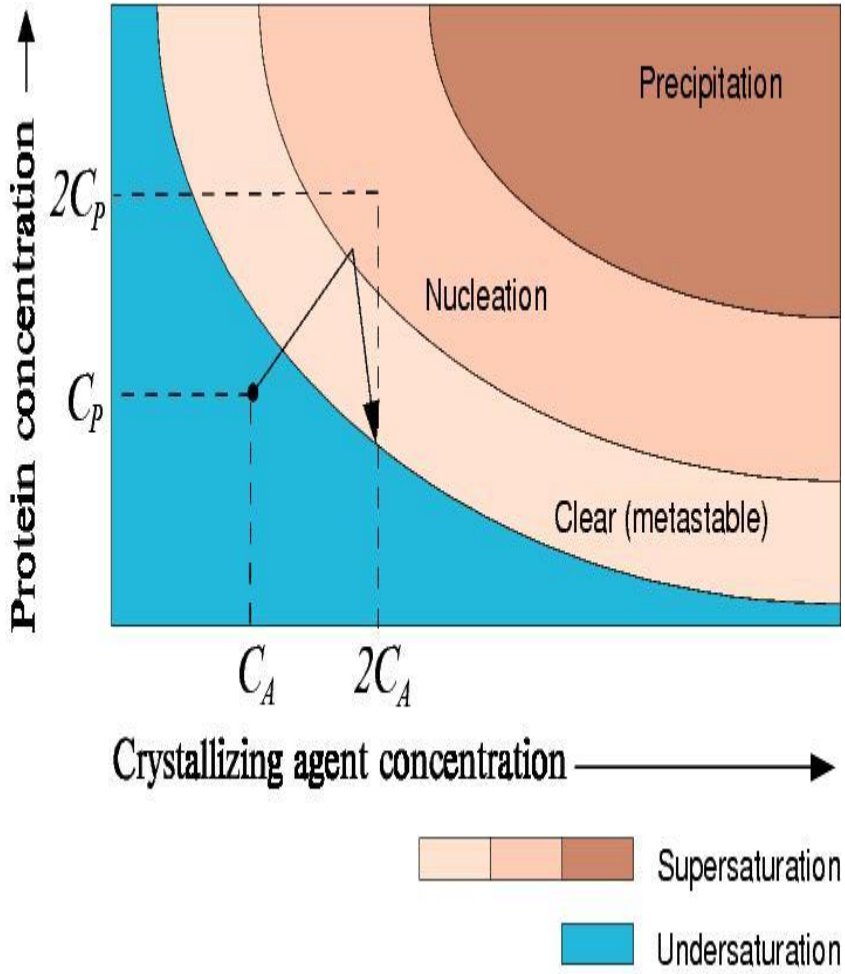
- Same basic principles as in hanging drop method, except
- the drop containing protein solution and precipitant solution sits on a bridge within the well.
- This allows for a larger sample size (20 - 40 μL), however
- Protein is very precious to the crystallographer, so, there is no much demand for larger volume of protein.

Phase Diagram for Vapor Diffusion

(No crystals)



(Crystals!!!)



Airlie J McCoy, Protein Crystallography course
http://www-structmed.cimr.cam.ac.uk/Course/Crystals/intro.html

Batch Crystallization

- This method involves mixing the biological macromolecule or protein solution and the crystallizing/precipitant solution to achieve supersaturation instantaneously.

- Since experiment starts at supersaturation – nucleation tends to be too large

- Large crystals can be obtained when working close to metastable

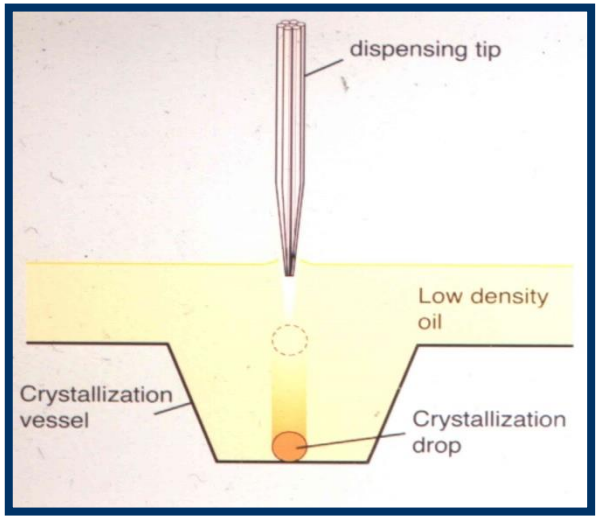
Microbatch Crystallization

- A miniaturized batch method where crystallization samples are dispensed as small drops (can be less than 1μl final volume) under oil.

- Enables systematic studies on very small quantities – μl scale, of both protein and crystallizing agents.

Applications include:

- Screening
- Optimization
- Control of nucleation



Chayen (1997) Structure 5, 1269-1274

Microdialysis

- Biological macromolecule is separated from the crystallization reagent by a semi-permeable membrane which allows small molecules (such as ions, additives, buffer etc.) to pass through but prevents the passage of the macromolecule

- Kinetics of the equilibrium depends on the membrane cut-off, the ratio of the concentration of crystallizing agent on either side of the membrane, the temperature and design of dialysis set up

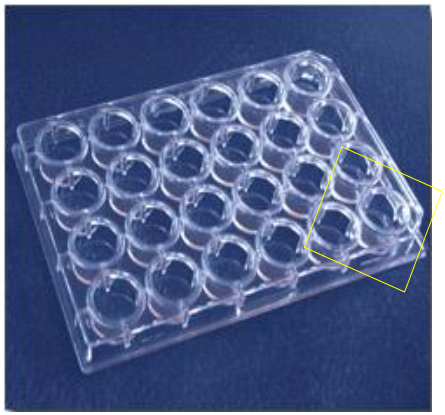
Free Interface Diffusion

- Also known as the liquid/liquid diffusion method
- Brings together protein and crystallizing reagent or precipitation solution without premixing them
- Each is injected through either sides of a channel, allowing equilibrium through diffusion.
- Both solutions come into contact in a reagent chamber, at their maximum concentrations, initiating spontaneous nucleation.
- As the system comes into equilibrium, the level of supersaturation decreases, favouring crystal growth

Methods of Crystallization Screening

Manual crystallization screening of crystals

a)



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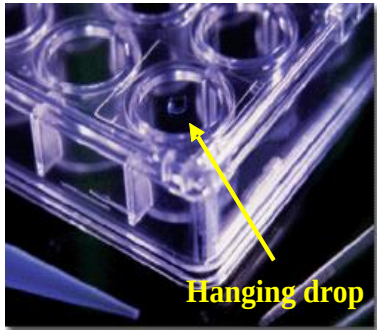

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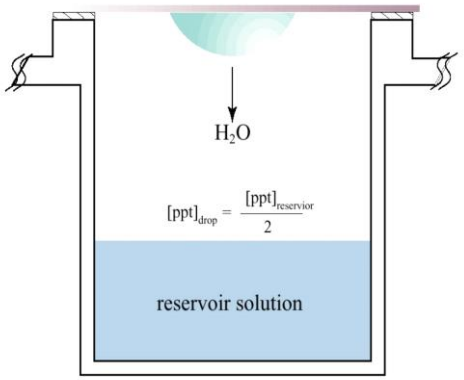
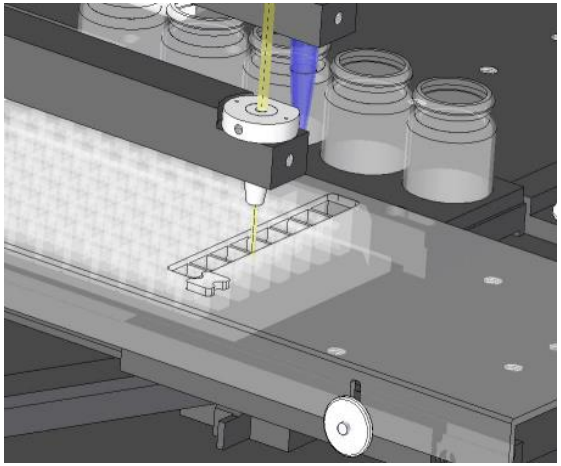


A robot for automated crystallization screening



Screening Strategies

Since it is impossible to predict the conditions for nucleation, screening is a good way of determining the crystallization conditions.

Random screens

Trial and error sparse matrix approach

Systematic screens

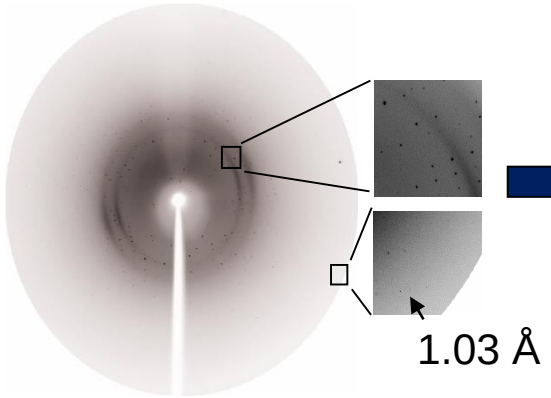
Selected variation of two parameters

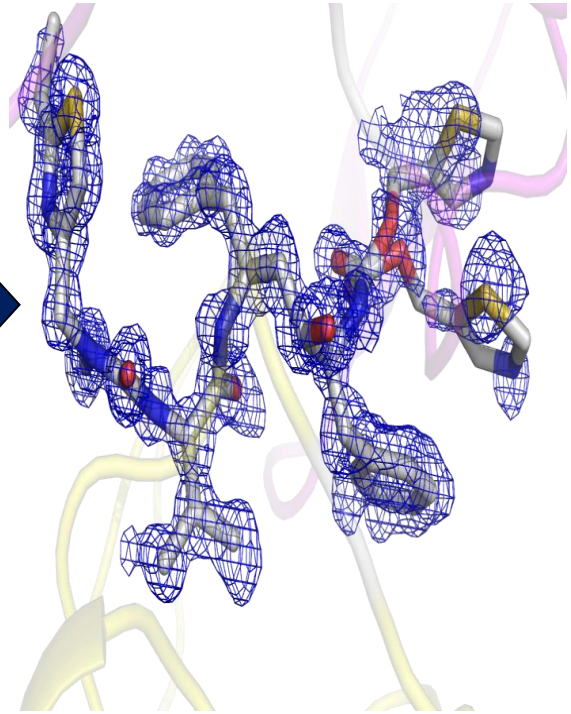
Sparse matrix screens

- Composed of a collection of conditions which have been used successfully for crystallization of other proteins
- Within the screens the following parameters are varied: pH, precipitating agent, type of buffer and salt components

Concluding Remarks

Cryo-loop





. .Success on your Biocrystallography Journey

Questions ???