Crystallization

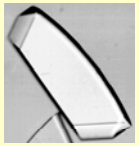
Lewis & Clark Workshop #1

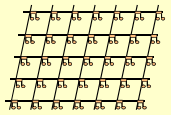
Why crystals?

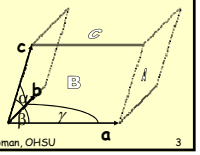
- Electrons scatter x-rays inefficiently (1 in 10^{16})
- Dataset from one molecule ~ 100 trillion yrs
- Solutions – average of all orientations
- Solution scattering provides dimensions
  - Overall shape from moments of inertia
  - Radial density function
  - Not detailed structure

Crystals are molecular lattices / arrays

- Crystals are arrays of ~ 10^{15} molecules with same orientation.
- Variation in orientation 0.2 – 1.5°.
- Scattering depends on direction
  - Structural detail







Crystallization

- Art; not completely scientific.
- Only partially understood.
  - Thermodynamics
  - Phase diagrams
  - Empirical understanding – what works
    - … usually

What's important to crystal quality?

1. Purity
2. Purity
3. Purity

- Protein repeated exactly at each lattice point
  - Don't want something else substituting…
- Biochemically pure – goes w/o saying
  - Want > 99% purity; No chance if < 97%
  - Note – 1-3% contaminants difficult to detect
- Need more than biochemical purity
  - Identical conformation.
  - Same post-translational modification.
  - Same proteolytic state.
  - Same chemical modification; eg. phosphates.

Crystals grow in 2 steps:

1. Nucleation - first aggregation.
2. Growth.

- Thermodynamically distinct
- Want a few nuclei to grow big
- Use thermodynamics to understand the required conditions

Supersaturation

- Solution at concentration > solubility
  - If at equilibrium → solid
  - But not at equilibrium
- All macromolecular crystals grown from superstaturated solutions
- Crystallization through controlled equilibration

Thermodynamics

- ΔG_g = free energy of germination. Ideally...
- $\Delta G_g = -kT(4\pi r^3/V)\ln \beta + 4\pi \gamma r^2$
- k = Boltzman constant.
- β = Supersaturation.
- r = radius of nucleation.
- V = volume of molecule in crystal.
- γ = interfacial free energy: crystal vs. solution.

Thermodynamic implications

- $\Delta G_g = -kT(4\pi r^3/V)\ln \beta + 4\pi \gamma r^2$
- Nucleation – start of crystal growth
  - Small radius - 2nd term dominates.
  - At low supersaturation (β)
    - Positive – ΔG is unfavorable
  - High supersaturation needed to start crystal
- Growth beyond critical size
  - Large radius – 1st term dominates
  - Always favorable

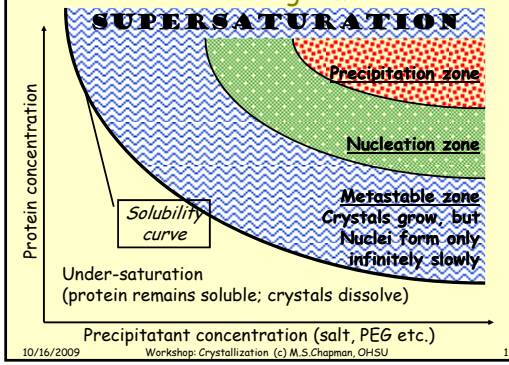
Implications for size

- $\Delta G_g = -kT(4\pi r^3/V)\ln \beta + 4\pi \gamma r^2$
- To maximize size
- One (a few) nuclei
  - to which all available protein added
- Want minimal β that only just nucleates
  - Finely tuned conditions
  - Experimental design
    - Initial β is non-nucleating
    - Slowly increase supersaturation

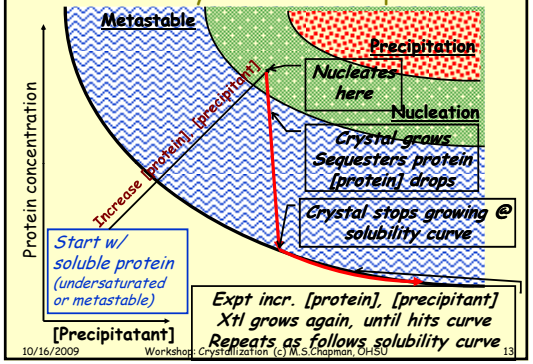
Crystallization vs. Precipitation

- Molecules identically oriented on regular lattice
- All molecules in optimal orientation/position
- Can occur at lower supersaturation
- Irregular aggregation
- Molecules joining before they can find the optimal position/orientation
- Occurs at high supersaturation
- Occurs when supersaturation increased quickly

Phase diagrams



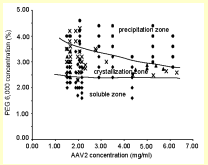
Course of Crystallization Experiment



Experimental Determination of Phase Diagrams

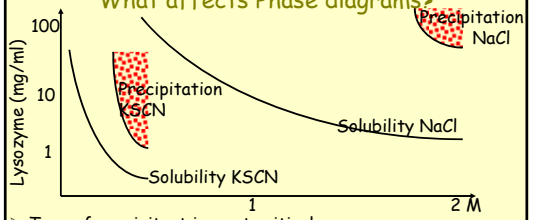
- Solubility curve at point when crystals dissolve
- Requires large supply of crystals
  - Only after you know how to crystallize
- Not much help in planning…
- Requires so much protein that determined only for a few proteins.
  - These phase diagrams are useful for other proteins

Generic phase diagrams



- General shape used to interpret experiments
- Plot conditions
  - → precipitation
  - → nucleation
- Try to separate the phases w/ typically-shaped solubility curve
  - ➔ Better guesses → trials that might → crystals
- With more & more trials, improve phase diagram

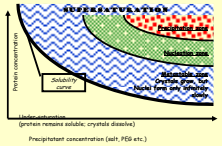
What affects Phase diagrams?



- Type of precipitant is most critical
  - Type of ion affects solubility
  - Need to try many
- Lysozyme: both supersaturation and precipitation occur at higher NaCl concentrations than KSCN
  - -> wider crystallization window for NaCl.

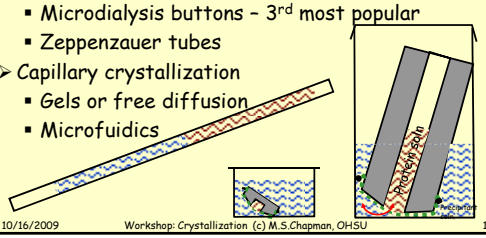
Thermodynamics of Phase Diagrams

- More nucleation @ high supersaturation.
- Supersaturation drops as crystals sequester protein
  - less nucleation
  - A few large crystals.
- Continued growth, requires increased supersaturation
- Experiment needs to increase [protein] and/or [precipitant] (usually both)
  - Dialysis or vapor diffusion
  - Slow enough so [protein] drops w/ Xtl growth
    - Avoids further nucleation



Methods for slowly increasing supersaturation

- Vapor diffusion:
  - Hanging drops – most popular
  - Sitting drops – esp. robotic setup
- Dialysis:
  - Microdialysis buttons – 3rd most popular
  - Zeppenzauer tubes
- Capillary crystallization
  - Gels or free diffusion
  - Microfuidics



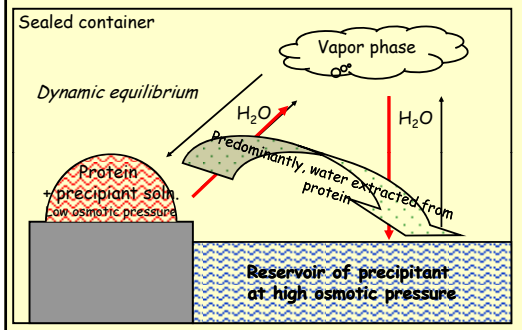
Preparation for crystallization

- Remove dust
  - Promotes excessive nucleation
  - Sterile filter (.22μ m).
  - Micro-centrifuge (10 min x 10,000 g)
- Prepare in stable buffer
  - To be incubated for months
  - Azide to inhibit fungi
  - Consider protease inhibitors

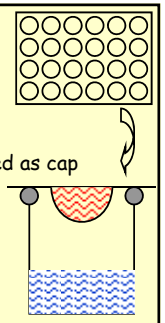
Dialysis methods

- Stretch dialysis membrane over capillary.
- Attach w/ tubing.
- Fill w/ (micro/Pasteur) pipette 10 – 300 μL.
- Place in precipitant solution (1 – 3 mL) & Seal
- Wait weeks for crystallization.
- Inspection difficult
  - Not well suited for screening
- Appropriate for large crystals w/ known conditions
  - When you have a bunch of protein

Principles of Vapor Diffusion



Hanging drops – most popular



- 24 (or 96)-well culture plate
  - Test many conditions
- Each – like mini-beaker
  - 1 ml precipitant in "well"
- Microscope cover slip (or tape) used as cap
  - Sealed on w/ vacuum grease
- Protein drop hangs from coverslip
  - 4 to 20 μL
- Advantages
  - Small scale
  - Approaches equilibrium slowly
  - Crystals seen thro' cover-slip w/ microscope

Hanging drop protocol

- (Siliconize cover slips).
- Grease rims of wells.
- Put 1.0 ml precipitant solution in each well.
- Prepare protein at ~1/2 precipitant concentration
  - Pippete 10μL protein solution to cover slip.
  - Add 10μL precipitant solution.
- Invert cover slip over well & seal.
- Carefully store plate.
- Inspect w/ microscope.

Crystallization depends on…

1. Purity
2. Type of precipitant
3. Concentration of precipitant
4. pH
5. Protein concentration
6. Temperature
7. Ionic strength
8. Additives at low concentration
   1. Ions, esp. divalent
   2. Ligands, coenzymes
   3. Detergents
      - (membrane proteins)
   4. Organic co-precipitants

- Other factors → success
- Reducing agents
  - DTT, βME
- Chelation of unwanted ions
  - EDTA?
- Denaturants (low conc.)
- (Dust-free; vibration free; controlled temperature)

Daunting combinations – Start w/ conditions effective w/ other proteins

- 1990 survey of precipitants:
  - 60% proteins crystallized with salts;
    - NaCl; $(NH_4)_2SO_4$; K_2PO_4.
  - 16% with organic solvents
    - Methyl-2,4-pentanediol (MPD)
  - 15% with PEG (polyethylene glycol).
    - Now, much more than 15%???
    - MW 2000 – 6000
    - slower than salt.
    - Single most effective precipitating agent

Clues from related proteins

- BMCD - Biomolecular Crystallization Data Base (NASA, NIST)
- Conditions copied from literature.
  - (With some errors!)
- Protein concentration, salt, PEG, pH...
- Look for your type of protein.

Screens (for when you haven't a clue)

- Derived from BMCD data base
- What 50 sets of conditions would get you close to the largest # of previously crystallized proteins?
  - Sung Ho Kim, Alex McPherson...
- Can purchase pre-made solutions, covering
  - Precipitants: salts, PEGs (var MW), MPD…
  - pHs – beware, some not as labeled
  - Additive ions
  - Organic co-precipitants
- Special screen kits for membrane proteins, immunoglobulins… many screens now available
- High chance of a lead – microcrystals etc.
  - Lead conditions need to be optimized

Automation and Robotics

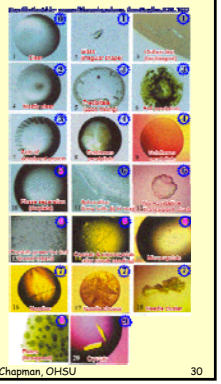
- Advantages
  - High throughput
  - Substitute for graduate students
  - Low volume
- Technologies:
  - Oil drop;
  - capillary microfluidics;
  - sitting / hanging drop
- Challenge
  - Pattern recognition
- Disadvantage
  - Cost: $50,000 - $1M+
- Xtal Biostructures Inc. & others – service $500-$2,000 (or free…?)
  - 10 μl to test several hundred conditions
  - Photographed daily for 2+ weeks

Automation components

- Drop-setting
  - Vapor diffusion
    - Hanging or sitting drop
  - Fast, accurate
  - Nanoscale (2-10 nL)
  - Pre-formed array of conditions
- Screens
  - Commercial arrays
    - $100s
    - Dozens available
  - Robotic liquid handling
    - $120,000
- Visualization
  - Microscope $10k + time
    - Polarization
  - + camera / xy-stage ($50k)
  - Multi-plate storage / robot - $120,000
  - Fluoresence
- Array optimization
- Integration – fully automatic - $1M+
  - Services...

Types of results - scoring

- Rarely get single crystals on 1st attempt
- Other results can indicate where to try next
- Some results more encouraging than others
  - Precipitates
    - Flocculent or granular?
  - Crystalline
    - 1D fiber, needle, plate…
- Various scoring systems



Assessing crystals

- Size (reticule)
- Perfection
  - Cracks / domains
- Protein?
  - Crossed polaroids
    - L-amino acids
  - Polaroid rainbows
  - Fluorescence
    - Optics
  - Izit ~ Coumassie
  - Softness

Improving crystals – Systematic screens

- Split each of N variables into S fine steps
  - E.g. [PEG] – 10 to 15% in 0.5 % steps
  - E.g. [Protein] – 5, 10, 15 mg/mL
  - pH in steps of 1.5 units
  - Additives
- Test one variable at a time
  - Perhaps a combination of conditions is required
  - May never see it
- Test combinations

Testing combinations – Factorial experiments

- Full factorial – all combinations – S^N.
  - Simplest - Perhaps when only one or two variables
  - Needs much protein
  - Many experiments
- Incomplete factorial
  - Random subset of all combinations
    - About $(NS)^2$ trials
  - Statistical analysis to indicate most important variables
  - more efficient

Kinetic considerations in crystal quality

- Nucleation rate: J_n:
- $J_n = B_s \exp(-\Delta G_g/kT)$
- B_s: product of solubility and kinetic parameter.
- Soluble protein: nucleates quickly (equilibrium).
- Less soluble: slow kinetics allows protein to be concentrated by dialysis or vapor diffusion.
  - Nucleation at high supersaturation
  - → shower of small crystals.
- Optimize solubility eg pH far from pI.

Optimal Rate of Crystal Growth is a balance

- Fast crystallization
  - takes place at lower supersaturation
    - →few big crystals.
  - → local concentration depletion
    - → crystal defects
- Control thro' solubility, drop size, temperature…
- Temperature complicated
  - $J_n = B_s \exp(-\Delta G_g/kT)$
    - Affects kT
    - Also affects solubility (up or down?)
  - Try experimentally 4, 20°C
- Large drop has lower surface-area:volume ratio
  - Slower equilibration by vapor diffusion

Conclusion

- Many things to try
  - One of the rate-limiting steps
- Good crystals greatly facilitate struct. Determin.
  - 1st crystals may not be the best possible
  - Optimize several types of conditions
- Read a good book before attempting crystallization
  - My favorite: Ducruix, A. and R. Giegé, Eds. (1999). Crystallization of Nucleic Acids and Proteins. 2nd Ed., The Practical Approach Series. Oxford Univ Press.