PETC Crystallization Discussion

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Sum Chan on Nov 16, 2007

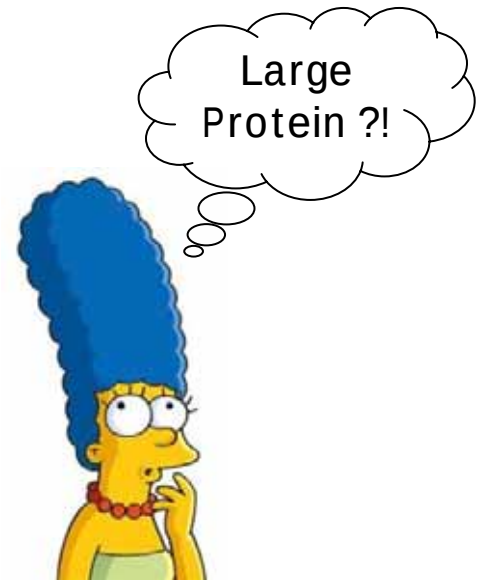
PETC Crystallization Discussion

Overview

- Things to do Prior to Protein Work
- Preparation of Protein for Crystallization
- Crystallization
- Strategies when Crystal is Absent
- Strategies when Crystal is Present

Before It Begins, Acquaint Your Target

- Molecular Weight
  - Larger proteins (>30kDa) are more likely to have multiple domains and may be more difficult to crystallize
  - But never say never, solving larger structure is definitely feasible
- Amino Acid Composition
  - 1 Met in every 100 residues for novel structure
  - Reasonable number of Trp for model building, UV microscope, and concentration determination
  - Free Cys can bind to Hg derivatives and help obtain the protein's electron density map

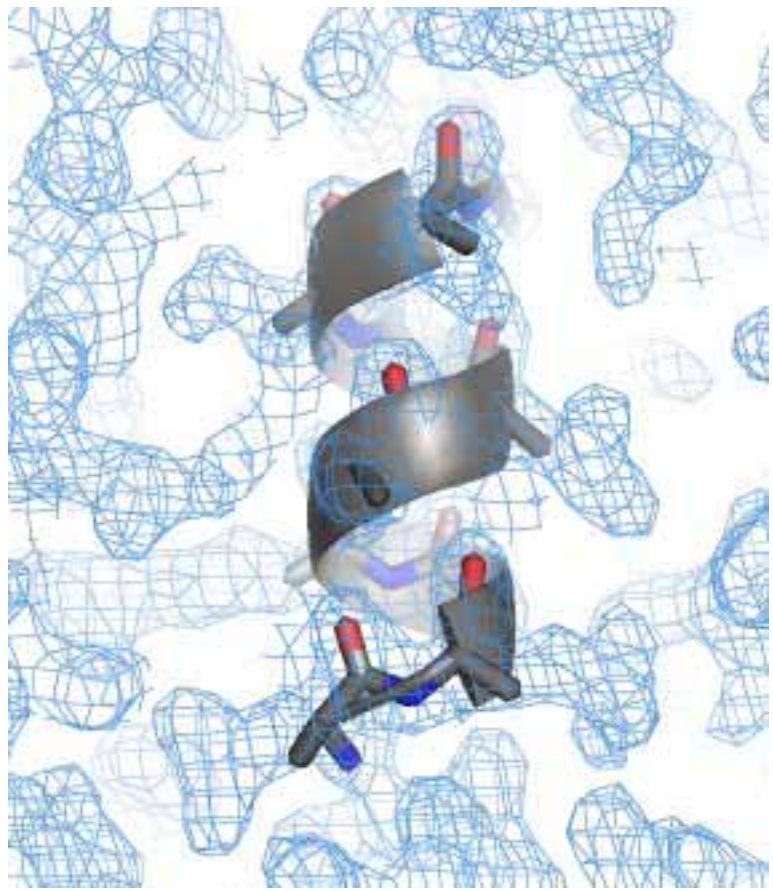


Before It Begins, Acquaint Your Target

- Isoelectric Point
  - Need to know for native gel and IEX
  - Important for solubility of protein – pH of buffer too close to pI of protein may cause precipitation
- Structural Homologs
  - Could help in calculations of the target's electron density map
  - Suggestions for target's function
  - Suggestions for target's ligand

Before It Begins, Acquaint Your Target

- Secondary Structure Estimate from Sequence
  - Estimated using sequence information and/or circular dichroism (CD)
  - Helps to judge the quality of the newly generated electron density map



Preparation of Protein for Crystallization

- Prerequisites
  - The protein has to be over-expressed and soluble
- High Purity
  - GF and/or IEX following IMAC for higher purity of protein prep
  - Pure sample allows more change for homogeneous molecules to interact with each other, thus better chance of nucleation of crystal
  - Purer the protein prep, the less contamination the protein crystal will have thus better chance of high quality crystal
  - Final storage buffer should be carefully chosen for crystallization

Preparation of Protein for Crystallization

- Protein Integrity Verification
  - SDS-PAGE
  - LC / MS for accurate results
    - Tells you whether the protein is full-length, Met-less, or truncated
    - Tells you whether the sample is homogeneous or heterogeneous
    - Tells you whether the protein is modified, e.g. carbamylation, oxidation
    - Tells you the polymeric state of the protein if the association between molecules is covalent

Preparation of Protein for Crystallization

- Protein Concentration

  - The higher the protein concentration, the higher chance it has for homogeneous molecules to interact with each other and higher chance for nucleation

  - Concentrate the protein using an Amicon Ultra-15 concentrator

- Choose the appropriate MW cutoff to avoid excess protein accumulation on the membrane → 5kDa MWCO usually works
- Centrifuge at 5000 x g for 1 to 4 hours depending on sample volume and viscosity
- Should have significant amount of NaCl in storage buffer to avoid hydrophobic interactions of the protein to the membrane

Preparation of Protein for Crystallization

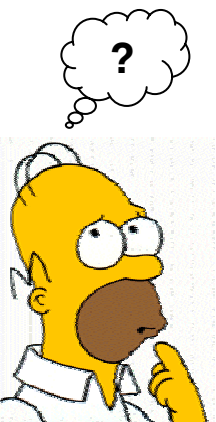
- Protein Concentration
  - Possible solutions when protein precipitates during concentration
    - Can try to set up a prescreen and see if it can be used for setting up crystal trays
    - Problem may lie in the buffer's pH, which should not be too close to the target protein's pI
    - Additive may be used to solubilize the insoluble (use microscope)
      1. Higher NaCl
      2. Small polar molecules such as glycerol
      3. Ligand if known
      4. Mild detergent, e.g. β-octyl glucoside, may break up "jelly"

Preparation of Protein for Crystallization

- Protein Concentration
  - Determination of the right protein concentration for crystallization
    - Before we had the prescreen
      - The magic number of 10mg/mL for protein ~30kDa
      - 20 to 50mg/mL for smaller protein
      - 2 to 5mg/mL for larger protein
    - Now that we have the prescreen
      - 4 to 6 medium precipitation → ready to go
      - Do Not forget your **negative control** during prescreening!
      - If your disbelieve the prescreen, set up one crystal tray (Hampton 1 & 2 or Hampton Index) and use that as the prescreen before setting up other crystal screens

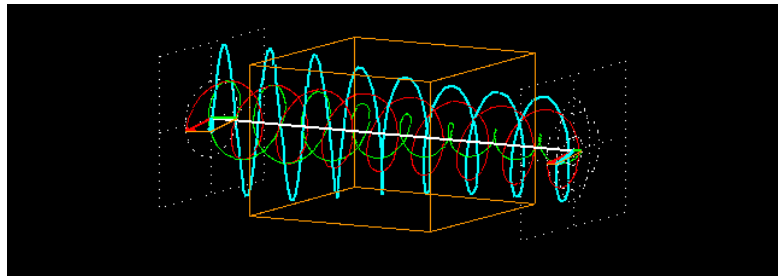
Preparation of Protein for Crystallization

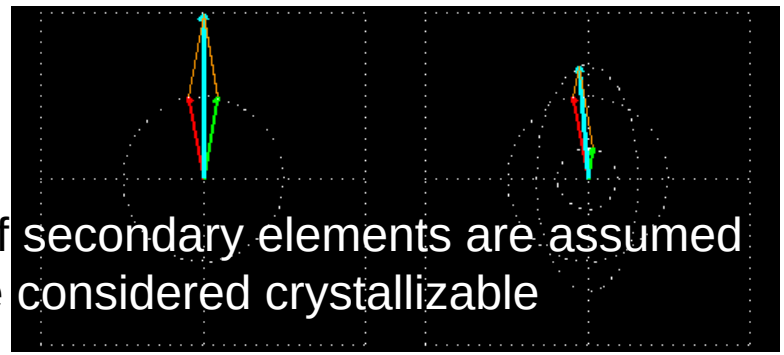
- Mono-Dispersity Determination
  - Mono-dispersity is as important as homogeneity
  - Native gel → to find out whether the sample is mono-disperse
  - GF → help estimate the polymeric state of the protein
  - Non-reducing SDS-PAGE → to find out if the polymer is covalently associated, i.e. forming intermolecular disulfide bonds



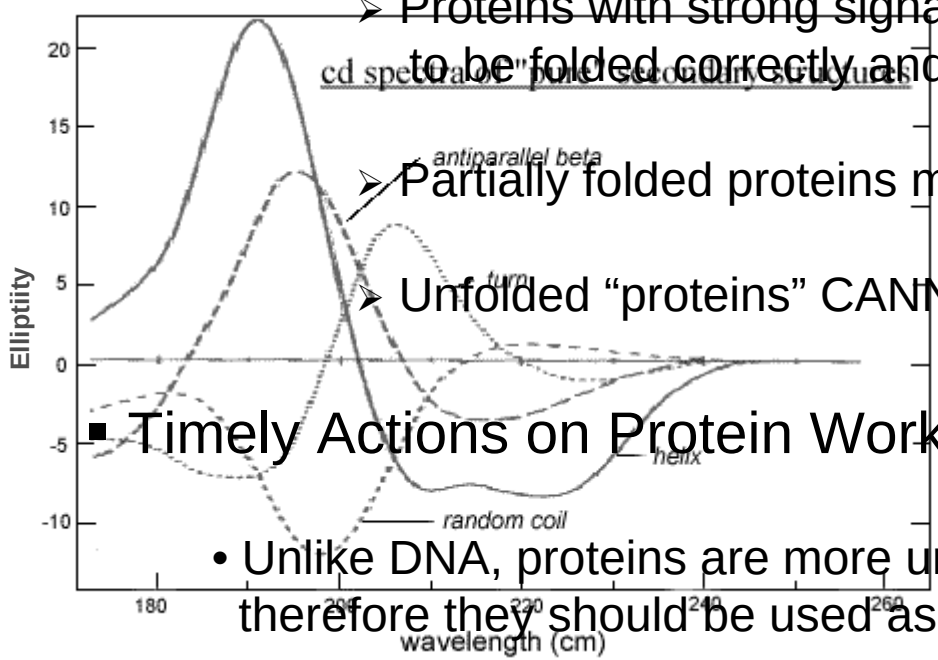
PETC Crystallization Discussion

Preparation of Protein





- Secondary Structure Presence
  - Circular Dichroism (CD Scans)
    - Proteins with strong signals of secondary elements are assumed to be folded correctly and are considered crystallizable
    - Partially folded proteins may be difficult to crystallize
    - Unfolded “proteins” CANNOT be crystallized



- Timely Actions on Protein Work!
  - Unlike DNA, proteins are more unpredictable and degrade much faster, therefore they should be used as soon as possible after purification!

Figure Source: http://www.huhaha.com/pages/cd.html

Animation Source: http://www.enzim.hu/~szia/cddemo/edemo14.htm

Crystallization Setup

- Crystal Drop Setup → Sitting Drop / Hanging Drop

- Crystallization Method → Vapor Diffusion

  - When the protein is added an equal volume of crystallization reagent, the components originally from the reagent are diluted by half in the drop.

  - In an air-tight environment, water from the crystal drop will try to go back to the reservoir in vapor form, in an attempt to balance the concentration on both sides. **1.5M NaCl** as reservoir are shown to have achieve similar effects.

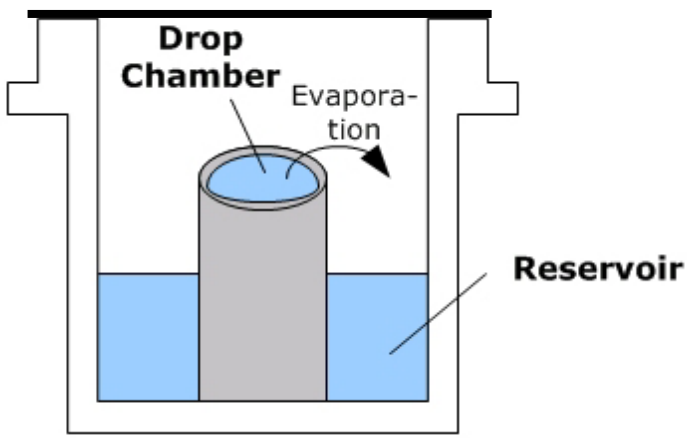


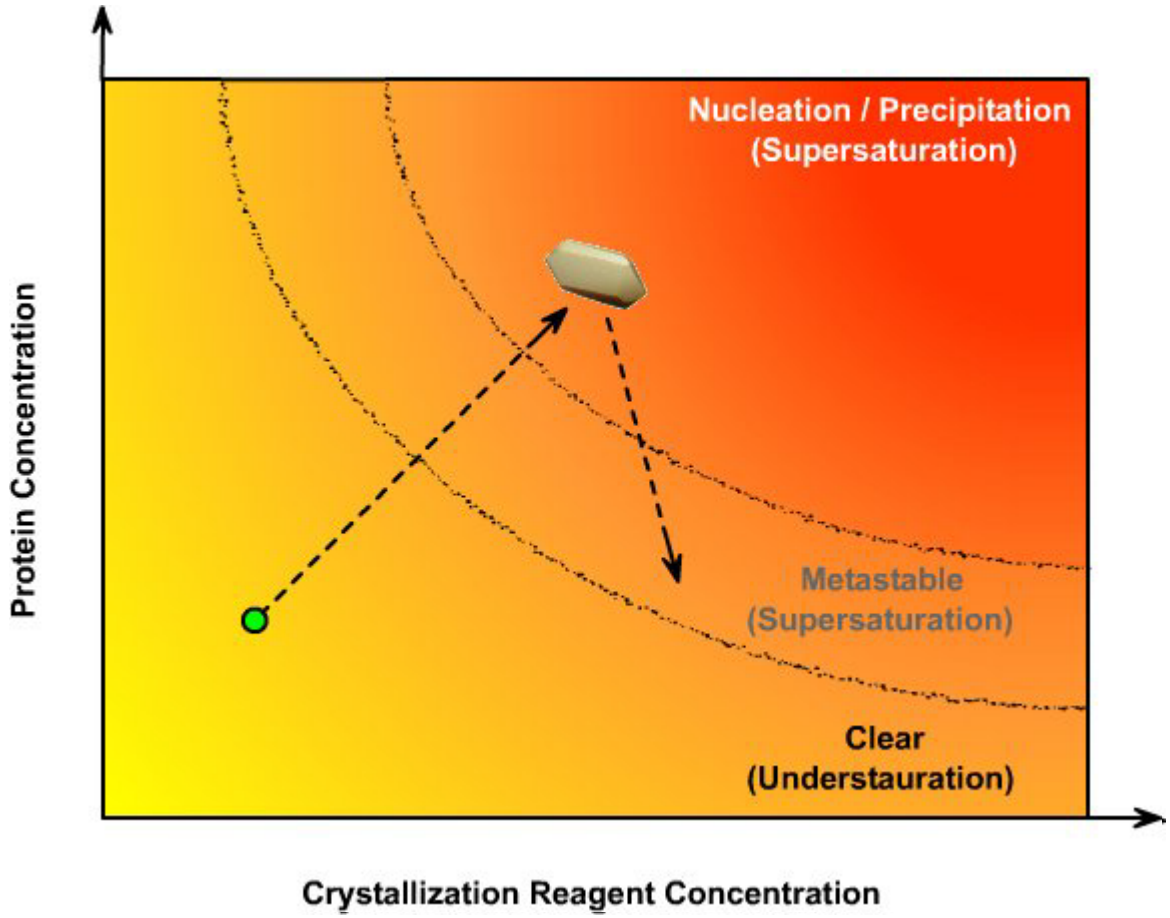
Figure Source: http://www.innovadyne.com/apps_crystallography.html

Crystallization Setup

- Commercial Crystal Screens
  - Hampton Screens 1 and 2
  - Hampton Index Screen
  - Hampton PEG/Ion and Cryo Screens
  - Hampton Natrix and Lite Screens
  - Hampton Salt Rx Screens 1 and 2
  - Emerald Wizard Screens 1 and 2
  - etc…
- Homemade Crystal Screen
  - Inna96

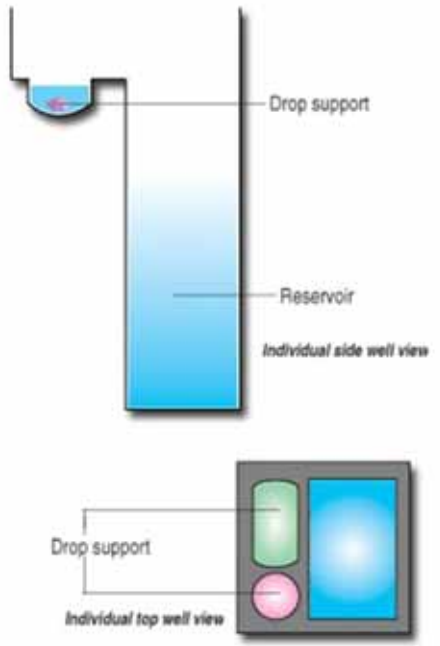
Crystallization Setup

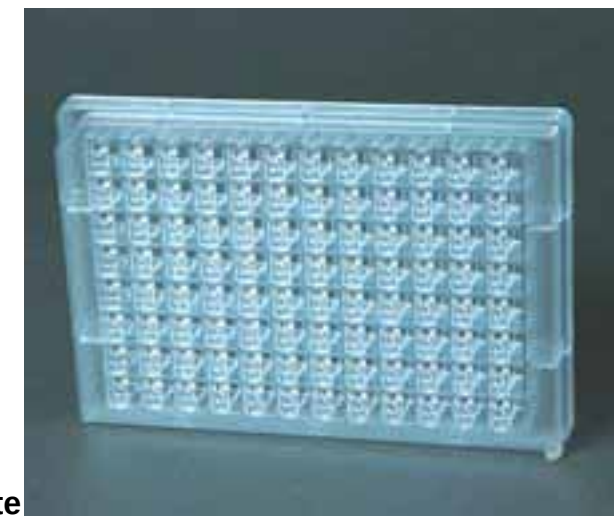
- Phase Diagram
  - Vapor diffusion is a slow process
  - Once crystal forms, protein concentration drop as the crystal grows



Crystallization Setup (Manual)

- Steps for Setting Up Crystal Tray
  - Transfer 100uL of 1.5M NaCl or crystallization reagent into the reservoir
  - Pipette 1uL of protein concentrate onto the drop support
  - Add 1uL of the crystallization reagent (NOT 1.5M NaCl this time!) onto the protein
- Cautions
  - Be very clean when using the crystallization reagent stock and cap the tubes tightly to minimize evaporation





Hampton 96-Well Intelli-Plate

Figure Source: http://www.hamptonresearch.com/products/ProductDetails.aspx?cid=10&sid=164&pid=82

Crystallization Setup (Manual)

- Cautions (Cont'd)
  - Take good care of the automatic multi-channel pipettes
  - Tight sealing of the tray is a VERY IMPORTANT step!

Crystallization Setup (Automatic, Almost)

- Cautions (Cont'd)
  - The "Mosquito" crystallization robot is helpful when the amount of protein is limited
  - Hanging-drop vapor diffusion is used instead of sitting-drop

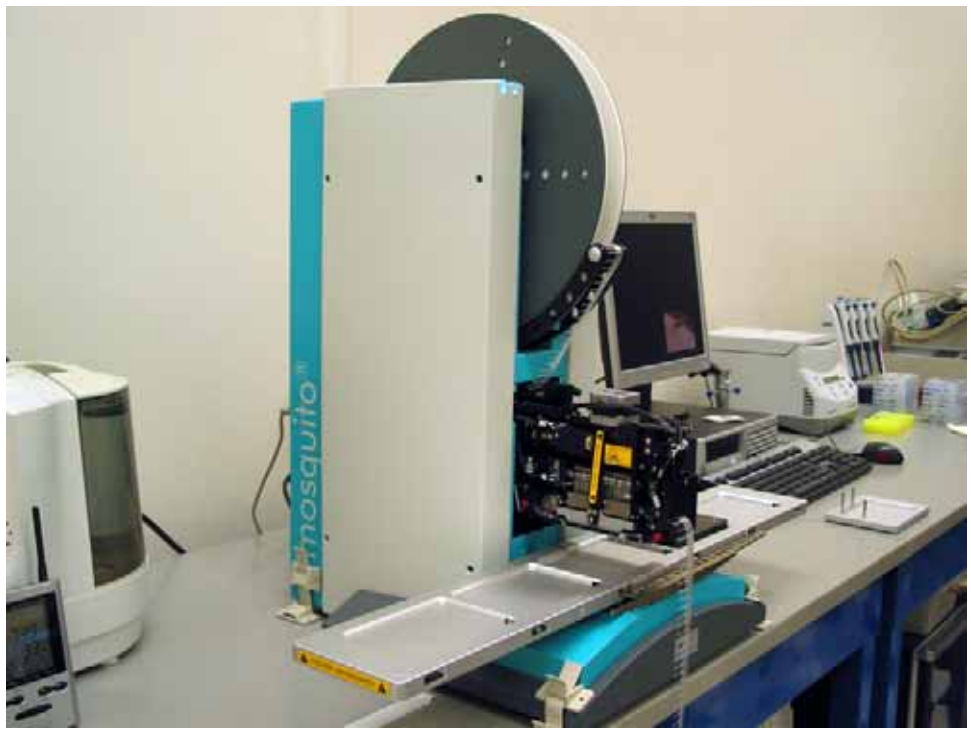


Figure Source: http://www.doe-mbi.ucla.edu/%7Ejnavarro/xtalscreens/mosquito.htm

Crystal Tray Observation

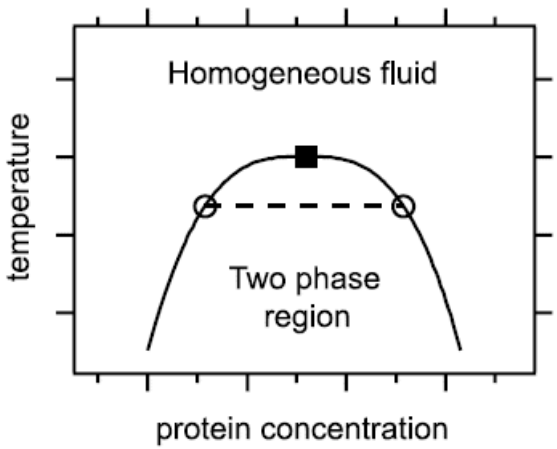
- How Often Should We Check the Trays?
  - **Immediately** (ideally)
    - Get an overall idea of the protein's behavior
    - Keep records of foreign objects
  - **The Next Day**
    - Fill in observation records
    - Useful for crystal trials that do not yield any crystal
  - **One & Two Weeks Later**
  - **One Month Later**
  - Whenever You Feel Like…

Strategies for Crystal Trials Without Crystal

- What If There's No Crystal Found?
  - Don't worry! There are many things to try!
- Too Few Precipitation and Too Light
  - Increase protein concentration by half
- Too Many Precipitation and Too Heavy
  - Decrease protein concentration by half

Strategies for Crystal Trials Without Crystal

- Large Number (>20%) of Phase Separation ("Oiling Out")
  - Liquid-Liquid Phase Separation (LLPS) is a narrow meta-stable transition in protein solution and is not often observed
  - Phase Separation could turn into crystals, and could be utilized to promote nucleation[1]
  - Double protein concentration, or raise incubation temperature, as long as protein stays folded
  - Add low concentrations (below C.M.C.) of various mild detergents → could be quite tedious
  - SER to mutate floppy residues to stabilize contacts between neighboring protein molecules for formation of a better crystal lattice



Phase Separation Diagram[1]

[1] Methods 34 (2004) p266-272

Strategies for Crystal Trials Without Crystal

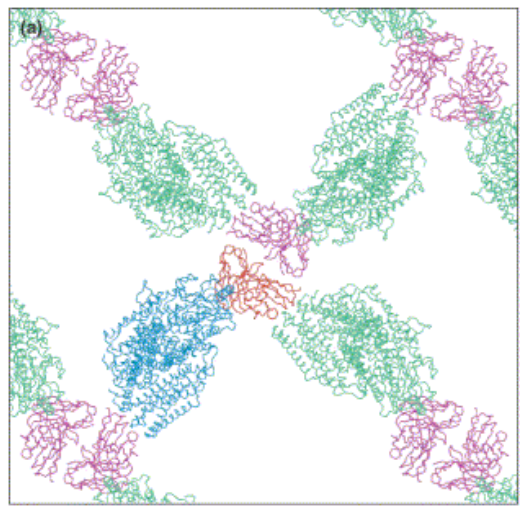
- Removal of Tag
  - Proteases such as Tev and thrombin may be used
- Redesign Construct to Relocate the Tag
  - Move the tag from one terminus to another
- Ligand Co-Crystallization
  - Crystallize the protein with known complex could alter its conformation significantly enough to form crystals

Strategies for Crystal Trials Without Crystal

- Temperature Change
  - Incubation at lower temperature slows down vapor diffusion and could grow more orderly crystals
  - Incubation at higher temperature may work for proteins originate from thermophilic organisms
- Co-Expression
  - Could reduce surfaces unfavorable to crystallization
- Truncation
  - Truncate the protein to reduce complexity (fewer domains) and entropy (possible floppy or hydrophobic regions)

Strategies for Crystal Trials Without Crystal

- Nucleation Inducer
  - Sonicated tiny pieces of horse hair is known to promote nucleations in crystallization sometimes
- Antibody-Mediated Crystallization
  - Providing additional surfaces for crystal contacts
- Work on Homologous Proteins
  - If one is desperate, could study the target's homolog instead, while working on the target

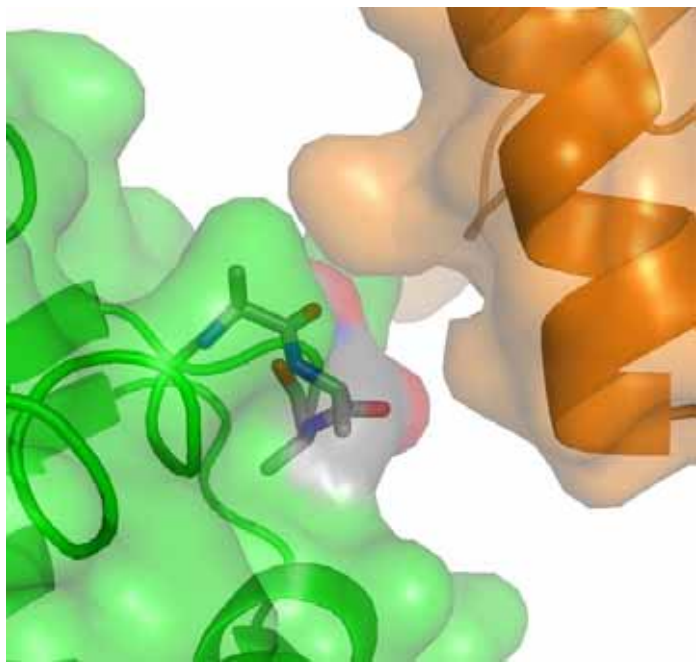


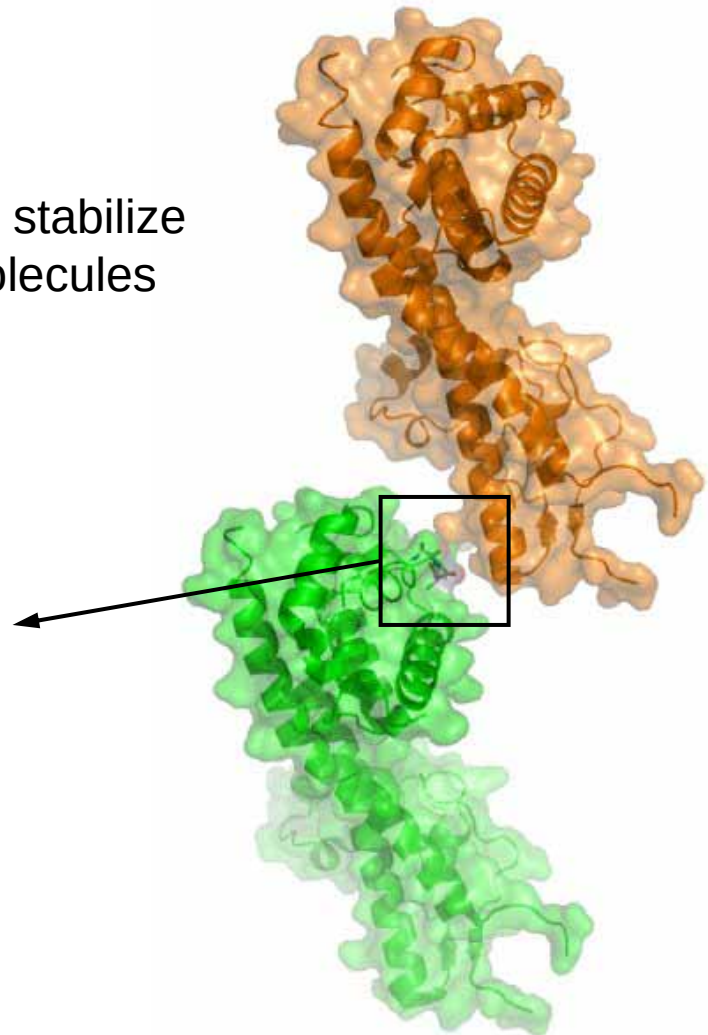
Cytochrome *c* oxidase crystallized with antibody fragment (Fv or Fab)

Figure Source: Current Opinion in Structure Biology, Vol. 12, Issue 4, Aug 1, 2002, p503-508

Strategies for Crystal Trials Without Crystal

- Surface Entropy Reduction
  - Mutate floppy surface residues to stabilize contacts between neighboring molecules

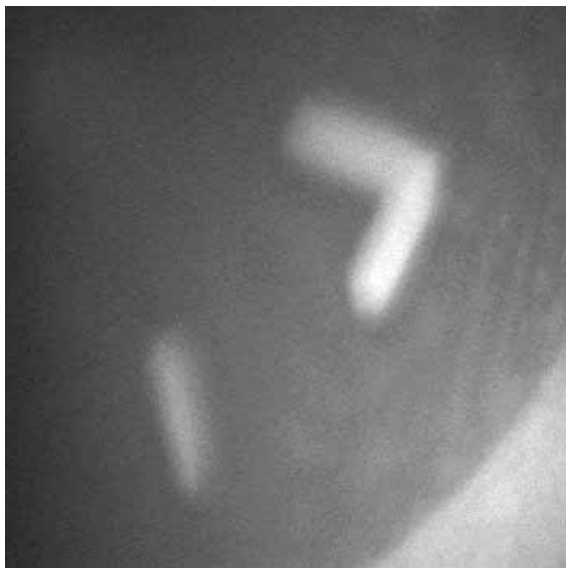


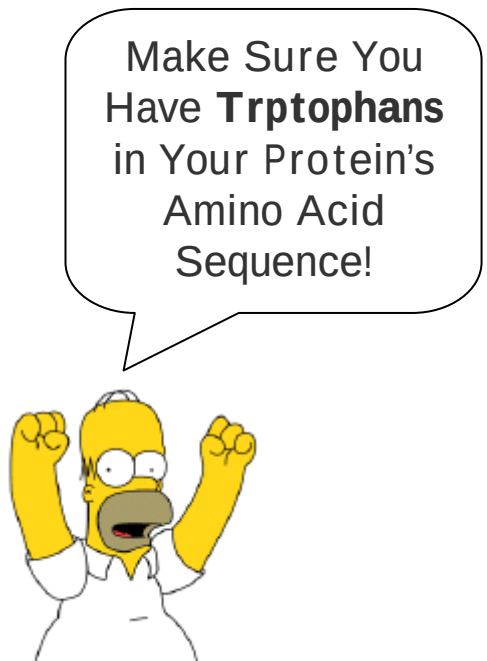


PDB: 1R6F

Strategies for Crystal Trials With Crystals

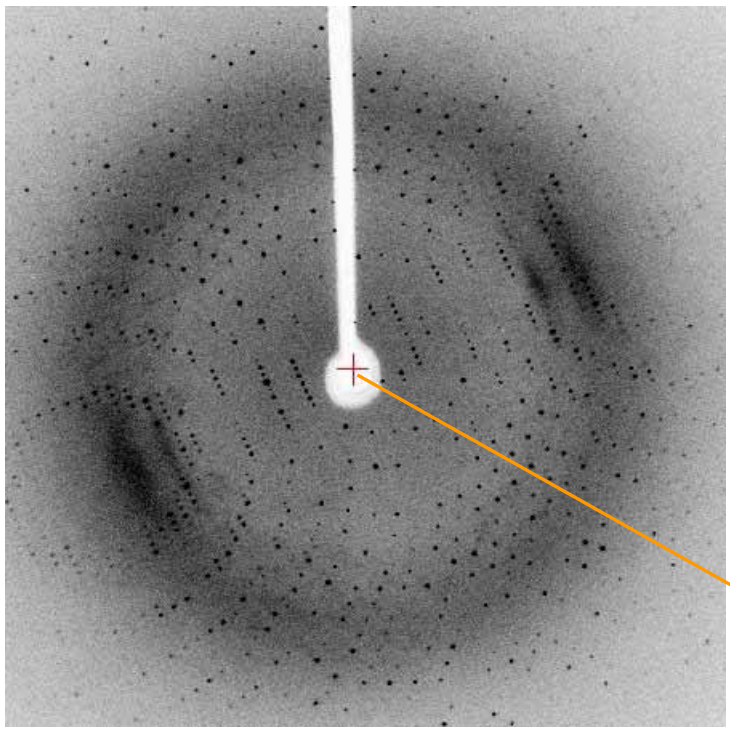
- What If Crystals Are Found?
  - Cheer!
  - Let Me Know! I'll make sure the crystals are made of protein!

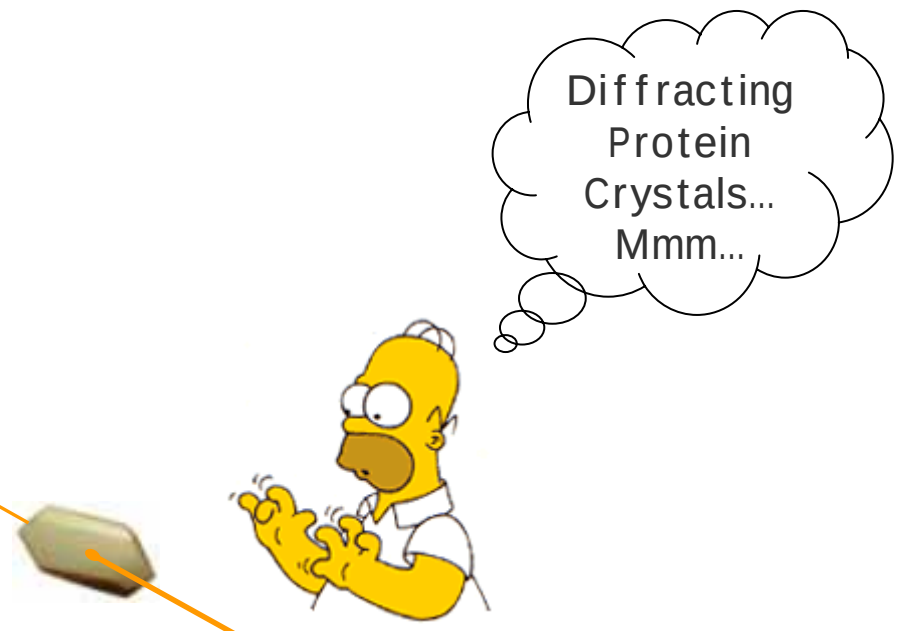




Strategies for Crystal Trials With Crystals

- What Kind of a Protein Crystal Do We Want?
  - A protein crystal that diffracts X-ray well





Strategies for Crystal Trials With Crystals

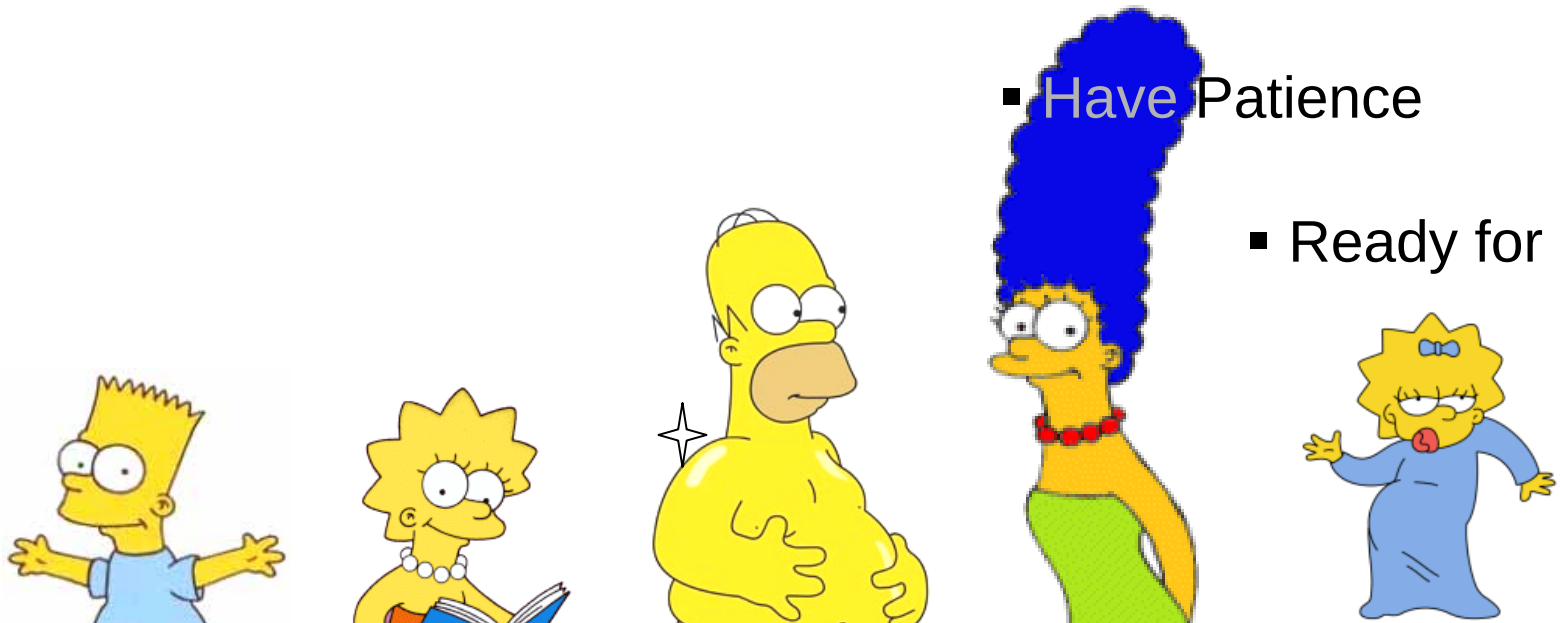
- What If The Protein Crystal Diffracts Poorly?
  - Post-crystallization treatments, e.g. dehydration
  - Grow better crystals → **Crystallization Optimization Trials**
    - Varying components of crystallization reagent that gave crystals
      - Precipitant concentration (often the major factor)
      - Salt concentration
      - Buffer concentration
      - Buffer pH
      - Type of buffer
      - Additive (find out from additive screens)
    - Growth crystals at different temperatures
      - Lower temperatures usually work better in these cases

Strategies for Crystal Trials With Crystals

- What If The Protein Crystal Diffracts Poorly?
  - Seeding
    - Microseeding – provides existing nucleation, which is a relatively high energy barrier for crystallization
    - Macroseeding – may not very due to imperfection of crystals
  - Removal of Tag
  - Ligand Complex Crystallization
  - Surface Entropy Reduction

Final Crystallization Tips

- Keep a Positive Attitude
- Work Hard & Research
- Be Clean & Careful
- Have Patience
- Ready for Surprises



Thank You!