BSTR521: BioCrystallography

Protein Crystallization, Crystal CryoProtection and Crystal Annealing

January 2007
Wim G.J. Hol

Protein Crystallography

Figure Courtesy of Focco van den Akker
A major text on protein crystallization is:

“Crystallization of Biological Macromolecules”
Alex McPherson
Cold Spring Harbor Laboratory Press

Chpt 4: Some physical and Energetic Principles
Chpt 5: Practical Procedures for Macromolecular Crystallization
Chpt 6: Important Considerations in Macromolecular Crystallization
Chpt 7: Strategies and Special Approaches in Growing Crystals

Some older information can be found in:

In particular the following chapters:

Chapter 3:
"Theory of Protein Solubility"
T. Arakawa and S. N. Timasheff, pp. 49-76.

Chapter 4:
"Nucleation and Growth of Protein Crystals: General Principles and Assays"
G. Feher and Z. Kam, pp. 77-111.

Chapter 5:
"Crystallization of Macromolecules: General Principles"
A. McPherson, pp. 112-119.

Chapter 6:
"Use of Polyethylene Glycol in the Crystallization of Macromolecules"
A. McPherson, pp. 120-124.

Chapter 7:
"Crystallization of Protein by Variation of pH or Temperature"
A. McPherson, pp. 125-127.

Chapter 8:
"Crystallization in Capillary Tubes"

Chapter 9:
"Seed Enlargement and repeated Seeding"
**PROTEIN CRYSTAL GROWTH (II) - Literature**

Methods in Enzymology Vol 276

Chapter 2:

Chapter 3:
*“Inferences Drawn from Physicochemical Studies of Crystallogenesis and Precrystalline States”*  Madeleine Ries-Kautt and Arnaud Ducruix, pp. 23-59.

Chapter 4:
*“Membrane Protein Crystallization: Application of Sparse Matrices to the α-Hemolysin Heptamer”*  Langzhou Song and J. Eric Gouaux, pp. 60-73.

Chapter 5:

Chapter 6:
*“Second Virial Coefficient as Predictor in Protein Crystal Growth”*  A. George et al., pp. 100-109.

Chapter 7:

Chapter 8:
*“Using Cosolvents to Stabilize Protein Conformation for Crystallization”*  Rui Sousa, pp. 131-142.

Chapter 9:

Chapter 10:
*“Dynamic Light Scattering in Evaluating Crystallizability of Macromolecules”*  Adrian R. Ferre-D’amare and Stephen K. Burley, pp. 157-165

Chapter 11:
*“Two-Dimensional Protein Crystals in Aid of Three-Dimensional Protein Crystal Growth”*  Aled M. Edwards et al., pp. 166-170.

Chapter 12:
*“Reductive Alkylation of Lysine Residues to Alter Crystallization Properties of Proteins”*  Ivan Rayment, pp. 171-182.

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**How to make the most of your precious protein solution?**

**Protect your protein from damage**

- Use “Flash freezing” for Long-term protein storage  
- Use Protease Inhibitors
- Use DTT, TCEP against oxidation
- Find Ligands in the Literature
- Find Ligands by gel shifts  
  - ALWAYS check pH of every solution you add to your protein solution

**Use your protein solution efficiently**

- Use small volumes in your crystallization micro-experiments
- Use minute amounts of protein to explore precipitation properties of your protein (in a so-called “pre-screen”)
- Explore the effect of temperature on solubility
How to make the most of your precious protein solution?

Optimize your protein buffer

Dynamic Light Scattering (DLS)
Low polydispersity in DLS is highly correlated with crystal growth.
Low polydispersity is an indication that your protein is present as a well-defined assembly and not a mixture of different aggregation states.
So, testing different buffers (increasing salt, glycerol concentration, changing pH, adding additives, etc) makes sense.

Optimize your protein concentration
In particular when your protein is forming multimers it might be good to try as high a protein concentration as possible so that your solution contains multimers only and is not a mixture of monomers and multimers (watch also your size exclusion chromatogram for hints)

HEAVY ATOM COMPOUND NATIVE GELS
(“HAC GELS”)
Follow-ups:
HAC as Additive in Crystallization
HAC for Soaks in Crystal Mounting

Monitor positional shifts as a result of (presumed) HAC binding
How to make the most of your precious protein solution?

**Limited Proteolysis** Provides information about:

- **Flexibility of your protein**
  - Speed by which protein gets broken down
  - At different pH values
  - At different temperatures

- **Protective effects**
  - Of general additives like NaCl, glycerol, phosphate, etc
  - Of specific additives like inhibitors, substrates, ligands, metals
  - Of protein partners, antibodies
  - Of DNA, RNA

Design careful controls to check possible inhibition of your protease by the additives!

You might like to use more than one protease – trypsin, GluC, chymotrypsin, elastase, ...

**Limited Proteolysis**

Tbru018012AAA, R5679

<table>
<thead>
<tr>
<th>Time for proteolysis</th>
<th>4 Proteases</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 = 0 hour</td>
<td>T = Trypsin</td>
</tr>
<tr>
<td>1 = Proteolysis in 1 hour</td>
<td>Th = Thermolysin</td>
</tr>
<tr>
<td>24 = Proteolysis in 24 hours</td>
<td>S = Subtilisin</td>
</tr>
<tr>
<td></td>
<td>G = Glu-C</td>
</tr>
</tbody>
</table>

Key:

Proteins differ greatly in Protease-sensitivity.
The more stable the protein (or protein-ligand complex) the greater the probability of crystal growth.
Worth serious consideration

FOR A **NEW STRUCTURE ONLY PREPARE SEMET PROTEIN**......

**Reason:** there are few things as sad as having a nice diffraction data set from a native (i.e. no heavy-atom-containing sulfur-Met) crystal and having great troubles obtaining such a crystal again....

With a SeMet crystal you might as well have had a perfect structure *at once*!!

**Caveat:** sometimes SeMet protein is difficult to express, is hard to purify, does not wish to crystallize, does not diffract as well...

Principle of Protein Crystallization

- The general idea is:
  - induce supersaturation,
  - form a limited number of nuclei
  - increase the size of these nuclei

- However, nucleation is a very difficult process to control.
  - Usually, one aims for a few to a few dozen nuclei per crystallization micro-experiment.
  - But quite frequently a significant precipitate is formed quickly, Which means one needs to lower either protein or precipitate concentration.
  - Sometimes crystals do form from the initial precipitate but this is usually a slow process.
Factors Affecting Protein Crystal Growth

1. pH
2. Ionic Strength
3. Temperature
4. Concentration of Precipitant
5. Concentration of Macromolecule
6. Purity of Macromolecules
7. Additives, Effectors and Ligands
8. Organism source of Macromolecule
9. Substrates, Coenzymes, Inhibitors
10. Reducing or Oxidizing Environment
11. Metal Ions
12. Rate of Equilibration
13. Surfactants or Detergents
14. Gravity
15. Vibrations and Sound
16. Volume of Crystallization Sample
17. Presence of Amorphous Material
18. Surfaces of Crystallization Vessels
19. Proteolysis
20. Contamination by Microbes
21. Pressure
22. Electric and Magnetic Fields
23. Handling by Investigator

Precipitating Agents

- **Salts**
  - Diminish electrostatic repulsion between proteins
  - Promote hydrophobic interactions between proteins
- **PEGs**
  - Compete for water molecules with proteins
- **Organics**
  - Lower dielectric screening and increase electrostatic interactions
- **Combinations of the above**
  - In different concentrations each...
  - At different pH values...
  - **LEADING TO A NICE COMBINATORIAL EXPLOSION QUICKLY...**
Histogram showing the relative success of the most popular precipitation agents. PEG has now become the most popular reagent, with ammonium sulfate next.


Salts Used in Crystallization of Proteins

1. Ammonium or sodium sulfate
2. Lithium sulfate
3. Lithium chloride
4. Sodium or ammonium citrate
5. Sodium or potassium phosphate
6. Sodium or potassium or ammonium chloride
7. Sodium or ammonium acetate
8. Magnesium sulfate
9. Cetyltrimethyl ammonium salts
10. Calcium chloride
11. Ammonium Nitrate
12. Sodium formate

From: Alex McPherson
Solubility of hemoglobin in concentrated phosphate buffers as a function of ionic strength and temperature.

Note: Ionic strength $\mu$ is defined as: $\mu = \frac{1}{2} \sum C_i z_i^2$
Where $C_i$ is the concentration of the i-th ion present in the solution and $z_i$ is its charge. Summation is done for all charged particles present in the solution.

Histogram of those ammonium sulfate concentrations producing macromolecular crystals

PEG variations

- Polyethylene glycols (PEGs) are among the most frequently used crystal growth agents.
- The molecular weights range from PEG200 to PEG20,000
- Sometimes “monomethyl PEG” gives better results than PEG – sometimes worse.
- Be aware of potential differences in purity – and oxidizing agents – in different PEG bottles…

Solubility of various proteins in PEG-4000.

Measurements were made in 0.05 M potassium phosphate, pH 7.0, containing 0.1 M KCl

25 randomly selected proteins were “set up” by sitting drop vapor diffusion at pH 7.0 and otherwise identical conditions against PEG-200, -400, -1000, -2000, -4000, -8000, -10000, and -20000. After 12 weeks of incubation, the trials were scored for crystals.

Organic Solvents Used Crystallization of Proteins

1. Ethanol
2. Isopropanol
3. 1,3-Propanediol
4. 2-Methyl-2, 4-pentanediol (MPD)
5. Dioxane
6. Acetone
7. Butanol
8. Acetonitrile
9. Dimethyl Sulfoxide
10. 2, 5-Hexanediol
11. Methanol
12. 1,3-Butyrolactone, and many more such as:
13. 1,4-butane diol

Top twelve from: Alex McPherson

<table>
<thead>
<tr>
<th>Name</th>
<th>Dielectric Constants</th>
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</thead>
<tbody>
<tr>
<td>Formamide</td>
<td>100.50</td>
</tr>
<tr>
<td>Formic Acid</td>
<td>47.90</td>
</tr>
<tr>
<td>Methyl sulfoxide</td>
<td>45.70</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>42.60</td>
</tr>
<tr>
<td>Glycerol</td>
<td>42.50</td>
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<tr>
<td>Nitromethane</td>
<td>39.40</td>
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<td>Ethylene glycol</td>
<td>37.70</td>
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<tr>
<td>N,N-dimethyl formamide</td>
<td>37.60</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>37.50</td>
</tr>
<tr>
<td>1,3-Propanediol</td>
<td>35.00</td>
</tr>
<tr>
<td>Methanol</td>
<td>32.80</td>
</tr>
<tr>
<td>1,2-Propanediol</td>
<td>32.00</td>
</tr>
<tr>
<td>2,4-Pentanediol</td>
<td>25.00</td>
</tr>
<tr>
<td>Ethanol</td>
<td>24.30</td>
</tr>
<tr>
<td>Acetone</td>
<td>20.70</td>
</tr>
<tr>
<td>Propyl alcohol</td>
<td>20.10</td>
</tr>
<tr>
<td>Isopropyl alcohol</td>
<td>18.30</td>
</tr>
<tr>
<td>Butyl alcohol</td>
<td>17.10</td>
</tr>
<tr>
<td>Pyridine</td>
<td>12.30</td>
</tr>
<tr>
<td>Cetyl alcohol</td>
<td>10.34</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>6.15</td>
</tr>
<tr>
<td>Pentane</td>
<td>1.80</td>
</tr>
</tbody>
</table>

Histogram of those MPD concentrations yielding macromolecular crystals.

Table 5.1. Methods for attaining a solubility minimum

1. Bulk crystallization
2. Batch method in vials
3. Evaporation
4. Bulk dialysis
5. Concentration dialysis
6. Microdialysis
7. Liquid bridge
8. Free interface diffusion
9. Vapor diffusion on plates (sitting drop)
10. Vapor diffusion in hanging drops
11. Sequential extraction
12. pH-induced crystallization
13. Temperature-induced crystallization
14. Crystallization by effector addition
Histogram showing the relative successful used of the various methods for macromolecular crystallization. Currently, vapor diffusion methods are far more popular than any other approach.


Vapor Diffusion

• By far the most popular method
  – Step1. Mix (un)equal small volumes of protein solution and precipitant solution
  – Step2. Let the mixture equilibrate through the vapor phase against a significantly (but not ridiculously) larger volume of the precipitant solution – the “reservoir”

• Typical volumes for the protein drop are 0.3+0.3 to 5+5 microliter
• Typical reservoir volume 0.1 to 2 milliliter
• Typical protein concentration 10 mgs/ml
• Several variant techniques:
  – “Sitting drop”
  – “Hanging drop”
Volumes in the hanging drop typically: 1 µL protein solution plus 1 µL reservoir solution – but that can vary up and down by factor up to 10, and the ratio need not to be 1:1.

This is a nice slide but in actual practice it is hardly ever done this way any more (see a more up to date slide a little later).

But for mass production of the same crystals it might be (re)considered perhaps??
A simplified view of the principle of vapor diffusion.

Water molecules travel thru space until equilibrium is reached.

But in precise terms, thermodynamic equilibrium is reached when the “thermodynamic potential” of water, $\mu_{H_2O}$, is the same throughout the system.

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Vapor diffusion – sitting drop variant

Side view of Crystal Clear Strip showing the eight separate walls.

Sample droplet is dispensed into well on ledge.

Reservoir solution typically 100 μl.
Micro-batch

- Is a very simple procedure:
  - Step 1. Add protein solution to the reservoir
  - Step 2. Add the “precipitant solution” to the reservoir
  - Step 3. Seal reservoir.
- Typical volumes for the protein drop are 0.1+0.1 to maybe even 5+5 microliter
- Typical protein concentration 10 mgs/ml
- A variant gaining popularity:
  - Micro-batch-under-oil

Microbatch-under-oil

- Procedure in outline:
  - Step 1: pipette oil into a well
  - Step 2: pipette protein solution under oil
  - Step 3: pipette ppt solution under oil
  - Step 4: centrifuge plate for good mixing (not always)
- Its main advantage is that it lends itself nicely to robotization with the small volumes under oil not subject to rapid evaporation
- When using water-permeable oils one has an extra effect: the concentration of protein and ppt is increasing gradually over time
More recently the "free-interface diffusion" procedure is getting new attention

- Step 1: deliver protein to a reservoir – usually cylindrically in shape such as e.g. a capillary
- Step 2: deliver precipitate solution to reservoir

Its main advantage is that it allows different positions in the reservoir to have different changes in protein and ppt concentration over time – i.e. different rates of nucleation are explored

When using water-permeable container material one has an extra effect: the concentration of protein and ppt is increasing gradually over time
Free-interface Diffusion in Capillaries

"Free interface diffusion" in a capillary

Photographs of a Nalgene 870 PFA capillary containing crystals.

(a) A tube showing a combination of free-interface liquid-liquid and vapor diffusion crystallization techniques. On the left is 60 µl of a precipitant solution containing polyethylene glycol in phosphate buffer, while separately on the right is a droplet of precipitant and protein solutions (5µl each) in contact with each other.

(b) A close-up view of the crystallization droplet that contains yellow crystals of dihydrolipoamide dehydrogenase. One of the characteristics of crystallization using this technique in these tubes is the initial formation of crystals specifically on the meniscus rather than attached to the tube wall.

Free-interface Diffusion and Microfluidics

(E) Prototype protein crystallization chip
144 parallel reaction chambers


(A) Section of a device showing three pairs of compound reaction chambers. Control channels are filled with 20 mM Orange G (Aldrich). Buried control channels of the elastomer chip are separated from openbottom flow recesses by a 15-m elastomer membrane. Hydraulic actuation of the control channel deflects the membrane and pinches off the flow line, creating a fluidic seal. Containment valves (Upper and Lower) allow isolation of compound wells during incubation. (Scale bars, 1 mm.)

A robust and scalable microfluidic metering method that allows protein crystal growth by free interface diffusion.


Finally, with regard to the purity of a protein sample, never trust anyone*. No matter who the source, analyze and the protein sample yourself to insure that it is not so obviously heterogeneous to begin with that your efforts will be hopeless. Be skeptical of claims of purity unless you have seen the evidence yourself.

From: Alex McPherson

*including yourself (WH)
Walking through Phase Diagrams

Different Crystallization Methods

Vapor Diffusion

Each Individual Well a Unique Vapor-Pressure End Point
Classical Micro-Batch Under Oil
(Non-permeable oil and trays assumed)

Modified Micro-batch Under Oil
(Water-permeable oil)

Each well a fatal and dry End Point
Vapor Batch Diffusion Plate

Fluid Reservoirs
Such that a Defined Vapor Pressure obtained for all drops under oil.
(The closer to optimization the better – since then differences between drops smaller.)

Place Barcode Here

Microbatch Under Oil
(with Defined End Point)

In Vapor-Batch trays each well same vapor pressure end point
Free Interface Diffusion
(impermeable reservoir material)

Free Interface Diffusion
(permeable reservoir material: no defined end point)
(or air gap between two solutions in capillary: defined end point)

Equal Start volumes assumed

Equal Starting volumes assumed
Special methods

- **Dialysis**
  - Has the great advantage that the same protein solution can in principle be subjected to many different precipitant solutions – in particular when the crystals grown can be readily dissolved again
  - Is quite labor-intensive to set up

- **Changing pH**

- **Epitaxial Growth**
Small volume Dialysis in thin-walled glass capillaries
If crystals grow – can go straight into X-ray Beam (at Room temp)

Microdialysis buttons (Zeppezauer)

Vapor diffusion of a presumed volatile base from the dessicator reservoir into the capillaries with protein

Used for the crystallization of “papain”, a plant sulfhydryl protease, by Jan Drenth et al. in 60’s.

EPITAXIAL GROWTH

- Often thought of as a possible way to initiate nucleation
- The surface, however, has to match the repeat distances of at least one surface of the (unknown) protein crystal
- The surface also has to have the proper physical chemical and properties to induce nucleation of the protein
Crystallization: a multi-step process

- Step 1: Initial Screening
  - Determine lead conditions for crystallization
  - Using a “coarse screen” also called “random screens”

- Step 2: Optimization of “hits”
  - Optimize lead conditions to produce diffraction quality crystals by “optimization matrices around” initial hits

Actually:
Often many optimization-generations needed
Temperature Variation
A subtle way to obtain (much better) crystals
(Even 14°C can give different crystals than 20°C)

Room Temperature

Cold Room Temperature

Lori Anderson & SGPP

SEEDING
A splendid way to obtain (much better) crystals form poor initial crystals

• As “seeds” can serve:
  – (Way) too small crystals
  – Crushed larger crystals
  – Mutant protein crystals
  – SulMet crystals for SelMet protein, and vice versa

• Various seeding methods
  – Micro-seeding - such as “Streak seeding” – usually with a particular hair from a particular domesticated animal - often with a range of
MICROSEEDING

crush crystals → seed stock → dilution series → seed drop


STREAK SEEDING

A. Pick up seeds from crystal

B. Streak pre-equilibrated drop

C. Crystals grow along streak line

Macroseeding

A. Pick up a crystal from drop
- plunger
- syringe
- sitting drop well
- capillary
- seed crystal
- mother liquor
- inverted pot
- reservoir solution
- pre-equilibrated protein solution

B. Wash crystal repeatedly in stabilizing solutions.
- seed crystal
- stabilizing solutions
- sitting drop well
- inverted pot
- reservoir solution

C. Transfer crystal to pre-equilibrated drop
- capillary
- seed crystal
- pre-equilibrated protein solution


Lipoamide dehydrogenase – Bram Schierbeek
MACROSEEDING

Lipoamide dehydrogenase – Bram Schierbeek

MACROSEEDING

Lipoamide dehydrogenase – Bram Schierbeek
ADDITIVES
Another way to obtain better crystals form poor initial crystals

• The idea is that small-molecule ligands either:
  – stabilize the protein which usually means a significantly increased probability of obtaining crystals (the so-called “freezers”)
  – assist in forming crystal contacts (the so-called “glues”)

(Sometimes the same small molecule can serve as “freezer” and “glue”)

(Sometimes additives are also called “co-crystallants”)

ADDITIVES
Frequently used additives:
• Substrates and products
• Substrate fragments
  – e.g. ADP for an NAD-binding enzyme
• Metals
• Any Ligand or Inhibitor
• Detergents like β-octylglucoside
• Heavy-atom compounds
  – HAC-native gels can be useful
• Any compound!

(There are special “additive screens on the market”)
Co-crystallants and Crystallization

Pfal008421WES Native

Pfal008421WES Native plus Acyclovir

0.1 M Ammonium Sulfate
0.1 MOPS pH 6.5
25% PEG 3350
Drop size 1ul + 1ul

0.2 M Ammonium Sulfate
0.1 M HEPES pH 7.5
25% PEG 3350
2.1mM Acyclovir
Drop Size 0.4ul + 0.4ul

The effect of a known inhibitor

Specific Synthetic Ligands Change Crystals
(but in this remarkable case left cell dimensions the same!)

Native ASU
Cyclosporin Co-crystal - SAME(!!)ASU

Tcru 13382 A cyclophylin homolog: without & with Cyclosporin

Jonathan Caruthers & SGPP
LT B-pentamers & MDT crystal contacts mediated by seven co-crystallant molecules.


Do Not Underestimate E.coli!

Surprisingly frequently heterologous expression of proteins into E.coli results in ligands bound to the protein of interest!

Metal ions, like zinc, are frequent donations from E. coli.

In some cases the donated ligand can even be labeled with selenium!
6-pyruvoyl-tetrahydropterin synthase (PTPS)

PFAL004546 from SGPP

- Human homolog: 14% & 28% identity
- Subunit size: 173 residues
- Resolution: 2 Å
- Structure solution: MAD
- Structure: 3 subs/ASU, biological hexamer

SGPP & Juergen Bosch

6-pyruvoyl-tetrahydropterin synthase (PTPS)

PFAL004546 from SGPP

6-pyruvoyl-tetrahydropterin in 1.5σ density (magenta = Zn²⁺)

Zn and 6-pyruvoyl-tetrahydropterin at active site
Putative Methyl Transferase
LMAJ004091AAA – from SGPP

Human homolog: 36 % (81/224)
Subunit size: 250
Structure solution: MAD
Resolution: 1.9 Å
Structure: 1 subunit / ASU
Cofactor: S-Adenosyl-homocysteine

Putative Methyl Transferase
LMAJ004091AAA from SGPP

Anomalous Difference Fourier contour levels: showing without ambiguity that this is a Selenium atom

...S(e)-adenosyl-Selenohomocysteine...
ROBOTICS

• **Advantages:**
  – Less human errors
  – Easier record keeping
  – (Very) easy repeat use of protocols
  – Frees scientists for the challenging tasks
  – Smaller volumes possible

• Some types of crystallization methods lend themselves well for robotics:
  – The sitting drop method
  – The microbatch-under oil procedure

• A bit more cumbersome is:
  – The hanging drop method

• Not only the initial set-up needs to be roboticized, but also the **imaging** of the many drops to quickly see if crystals have appeared

• Also Preparation of Solutions can benefit greatly from Robotics

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The HTP Search Lab in Buffalo

a. The Robbins Scientific Hydra 384 pipetting robot
b. A 1536 well Greiner plate
c. The HTP robotic photo stand
d. Macroscope thumbnail photos
e. An enlargement of an interesting thumbnail, showing information about the crystallization cocktail.

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Initial Screening for Leads

*George DeTitta, February 2001*
SGPP Crystal Optimization Robots

Crystal Track Designs Matrices
Crystallographer Review Manual Scoring
Harvestable Crystals
Structure Determination Units

Hydra II Plus 1
Acapella

Database and Image Archive

RoboDesign MicroScope II

Larry DeSoto & SGPP

Crystallization Solution Maker

Birdfeeder

Birdfeeders on platform

ALCHEMIST (RoboDesign)
How to make the most of your crystals??

- A protein crystal can be VERY precious – in particular when only a few could be obtained!
- Procedures to obtain the most diffraction and phasing data out of your crystals:
  - Cryo-protect
  - Anneal
  - Dehydrate
  - Soak in Heavy-Metal Compounds
  - Save exposed crystals for SEEDING

CRYO PROTECTION

- Protein crystals suffer at room temperature from serious X-ray radiation damage
- Cryo-cooling the crystals to 100 K is a way to dramatically reduce the radiation damage
- The cooling process needs to avoid the formation of ice crystals in the protein crystal since these destroy the order of the protein molecules in the protein crystal
- Some “mother liquors” are amenable to cryo-cooling without any additions – many others need additives to save the crystals during the cooling process

CRYO PROTECTION

Fig. 1. Photograph of a flash-cooled crystal mounted in a fiber loop. The crystal was picked up with the loop (left side of the figure) from a drop of harvest buffer and flash-cooled in the nitrogen gas stream from a commercial cryostat. The loop was made by forming it around a wire support and twisting the free ends to form a long stem, which was then coated with glue to both stiffen it and prevent unraveling. The stem was cemented to a wire support (visible on the right-hand side of the figure), which was attached to a steel base (not shown). The loop diameter is ~0.33 mm.


Table 1. List of cryoprotectants used successfully in flash-cooling macromolecular crystals.

<table>
<thead>
<tr>
<th>Type</th>
<th>Concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>13–30 (v/v)</td>
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<tr>
<td>Ethylene glycol</td>
<td>11–30 (v/v)</td>
</tr>
<tr>
<td>Polyethylene glycol 400</td>
<td>25–35 (v/v)</td>
</tr>
<tr>
<td>Xylose</td>
<td>22 (w/v)</td>
</tr>
<tr>
<td>(2R,3R)-1-butanol-2,3-diol</td>
<td>8 (v/v)</td>
</tr>
<tr>
<td>Erythritol</td>
<td>11 (w/v)</td>
</tr>
<tr>
<td>Glucose</td>
<td>25 (w/v)</td>
</tr>
<tr>
<td>2-methyl-2,4-pentanediol (MPC)</td>
<td>20–30 (v/v)</td>
</tr>
</tbody>
</table>

The list was compiled from unpublished observations in the laboratories of Stephen Harrison and Don Wiley at Harvard University and a survey of structures and reports in six journals for the years 1993 and 1994. Forty different crystals are represented. The range of reported concentrations for each cryoprotectant is also given.

CRYSTAL ANNEALING

• Variations:
  – Flash Annealing
  – “New Solution” Annealing
  – Dehydration (& Rehydration)
  – Controlled Humidity Changes
    • at Room Temperature
      – Huber group

• General Ref:

FLASH ANNEALING

– In its simplest form: simply block the liquid nitrogen stream for around 30 seconds
– This allows the crystal to warm up
– Maybe the "crystalline mosaic" can reshuffle and reorder in this process
– The crystal attracts/looses water during this process so that changes in water content of the crystal might also be a factor

Procedure independently invented in many labs – first publication seems to be:
Glycerol Kinase Diffraction pattern - Prior to Flash Annealing

Flash Annealing

...and after annealing
The Power of Flash-Annealing

Literature examples:
post-crystallization treatments can improve
diffraction quality and resolution

Dehydration, cryocooling, annealing, rehydration
Sometimes “only” crucial improvement in mosaicity
Sometimes only “minimal” resolution improvement by 0.3 Å
Sometimes real significant resolution improvement by 1.5 Å
Up to 8 Å resolution gain reported…..
### DsbG: 10>2 Å

- Initial diffraction: 10 Å, streaky spots
  - Tried stepwise equilibration into cryo, annealing. ~90% solvent
- Dehydration method
  - Transfer crystal from crystallization drop (20% PEG 4K) to 5µl hanging drop of dehydrating solution (30% PEG 4K), equilibrated against 1 ml dehydrating solution overnight at 4°C
    - Treated crystals were more robust than untreated crystals
  - Equilibrated in 2 steps (10 min each) against dehydrating solution + 15% and 25% glycerol
- New diffraction: 2 Å, no more streaks! 53% solvent.


### EF-Tu-Ts

- Complex of 2 elongation factors from E. coli (EF-Tu is a guanine-nt-binding protein, EF-Ts is a guanine-nt-exchange protein)
- Initial diffraction: 5 Å, 61% solvent
- Dehydration technique:
  - Over 24 steps, exchange ML (20% PEG4000 + 90 mM (NH₄)₂SO₄) for cryo solutions with more concentrated PEGs and no (NH₄)₂SO₄
  - Crystals first developed cracks parallel to one crystal axis; cracks disappeared after 21st transfer step
- New diffraction: 2.7 Å, 55% solvent

NF-κB P52:DNA

- Eukaryotic transcription factor mediating immune response in mammals; important in expression of viral genes in HIV and herpes infections
- Initial diffraction: anisotropic, 2.4 Å x 3.4Å. 51% solvent
- Dehydrating method
  - Serially transfer crystal from ML (4-6% PEG4K) to dehydrating cryo (ML + 30% PEG400) + heavy atom (YbXX)
    - Shrinkage along one axis could be observed in crystal
- New diffraction: isotropic, 2.1Å. 49% solvent


HIV-RT:inhibitor

- Transcribes viral ssRNA into dsDNA for incorporation into host cell genome
- Initial diffraction: 3.7 Å
- Dehydration method
  - Transfer crystals to drops of increasing PEG3400 by 5% steps, from 6%-46%, over 3 days
- New diffraction: 2.2 Å

**P. aeruginosa PDF**

- Essential bacterial/microbial enzyme that modifies newly synthesized polypeptides, actively pursued as drug target
- Initial diffraction: ~2.0 Å, but poor reflection shape at high resolution
- Dehydration method
  - Flash frozen in 20% PEG 4000 (vs. 12% in ML) + 10% PEG 400
  - Removed crystal from cryostream and placed in 200µl drop of cryo without sealing for 30min, then flash-frozen again
- New diffraction: 1.85Å, with better spot shape. 50% solvent


**DsbC-DsbDα**

- Complex of a PDI (DsbC) and the transmembrane electron transporter (DsbD) that reduces the oxidized form of DsbC
- Initial diffraction: 7 Å, radiation-sensitive. 55% solvent.
- Dehydration method
  - 8x drop volume of cryo buffer (40% PEG5000 vs 25% in ML, + 10% glycerol) was slowly added to crystallization drop; cover slip was inverted and drop was exposed to air for 30 min before crystals were cryo-cooled
- New diffraction: 3.8 Å, 2.3 Å at synchrotron. 41% solvent

PDH – E2
Dehydration - Rehydration

- Pyruvate Dehydrogenase: E2 component forms a highly symmetric core around which E1 and E3 components assemble
- Initial diffraction: 7 Å from non-dehydrated drop, mosaicity=1.09°
- Rehydration method
  - Crystals from completely dehydrated drops (28 months after set-up) were resuspended in cryo (ML + 30% glycerol) and cooled in cryostream
- New diffraction: 4.2 Å, mosaicity=0.94°. 73% solvent


Take-home lessons annealing

- These methods worked for others; they may work for your problematic crystals as well
- Quick, proven methods:
  - Interrupt the cold stream for 20 – 30 – 45 seconds
  - cryo-ing with higher concentration of precipitating solution
  - leaving your cryo-ed crystal out to dry during coffee
- Slightly more labor-intensive, but gentler methods:
  - Equilibrating your crystal in a drop overnight against a dehydrating solution
  - Serially transfer your crystal from initial conditions to dehydrating conditions over time
- Even a dried-up drop could have useful crystals in it!!
- Don’t give up – dehydration and/or annealing is much faster to try than starting from scratch with a new construct!
Alternatives for obtaining better crystals

- **With current protein sample:**
  - Purify your protein better
    - Use Native gels in addition to SDS PAGE
    - Try iso-electric focusing?
  - If the protein of interest is nucleic acid binding:
    - DNA and RNA length variations

- **Back to molecular biology:**
  - Make smart mutations – but how
    - Derewenda et al
  - Make truncations or elongations
  - Try homologs from other species
  - Form complexes with partner proteins

- **With lots more work:**
  - Prepare antibodies – $F_a$’s or $F_v$’s
    - Phage display for generating specific antibodies
    - Classical hybridoma techniques

Modify your Protein
(And treat as a “new” protein in a new series of crystal growth optimizations)

- **Limited proteolysis**
  - Usually one uses trypsin varying:
    - Ratio trypsin to target protein
    - Temperature
    - Time
  - Can remove floppy tails, or indicates stable domains, and give immediately useful protein
    - Quite rare that that happens
    - Usually a fragment has to be characterized and re-cloned

- **Lysine Methylation**
  - Appears to give a remarkably uniform preparation

- **Tyrosine Iodination**
  - Not certain if it results in a homogeneous preparation
Protein Engineering for obtaining better crystals sometimes even AFTER STRUCTURE HAS ALREADY BEEN SOLVED

Reasons:
– Difficulties in protein expression
– Marginal protein solubility
  – Change surface side chains from phobic to phyllic
– Crystals of poor or undesired quality due to
  • Growth highly irreproducible
  • Twinning
  • Poor resolution
  • Anisotropy
  • Inaccessibility of active site
    – Change crystal contact residues

P. falciparum Peptide Deformylase (PfPDF)
A case study

Initial Structure Determination with crystals which:
1. Were very anisotropic with 2.8/4.0-ish Å resolution
2. Required multiple rounds of annealing
3. Contained 10 subunits per asymmetric unit
Ten copies of Pf PDF in the asymmetric unit

PfPDF constructs for altering crystal contacts

Original

Re-engineered

(6 residues: improved expression)

(3 residues (A-E-R-P): unfavorable crystal contacts)
### PfPDF

**various crystal forms obtained**

<table>
<thead>
<tr>
<th>Protein Variant</th>
<th>Inhibitor</th>
<th>Space group</th>
<th>Cell dimensions (Å)</th>
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<td>P4₁</td>
<td>a=b=121.3 c= 177.3</td>
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<tr>
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<td>P₆₅</td>
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<td>P₃₁,₂₁</td>
<td>a=b=65.1 c = 82.7</td>
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<tr>
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<td>P₄₁</td>
<td>a=b=76.0 c = 155.1</td>
</tr>
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</table>

Abhinav Kumar, Mark Robien, Brian Kramm, Bjarni Ingason

### RNA Editing TUTase 2

**Surface Mutagenesis Essential**

**Mutation potential surface exposed bulky residues to Arg on TbTUTase**

<table>
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<th>NO</th>
<th>Mutation</th>
<th>Construct</th>
<th>Expression</th>
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<td>OK</td>
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</tr>
</tbody>
</table>

**Quite clear where RNA binds**

- Combine the best

1) 94/247 R
2) 122/247 R
3) 94/122/247 R
4) 442/247 R
5) 94/122/247/442 R

Deng, EMBO J. 24, 4007-4017 (2005)
Crystals can vary GREATLY in Solvent Content

(A) The plot of unit-cell volume versus mass (in daltons) of protein in the unit-cell made by Matthews (1968) for 116 protein crystals whose cells had been characterized by X-ray crystallography.

Almost all were obtained using salting out procedures at high ionic strength.

The mean value for a crystals of the ratio of unit-cell volume to mass, known as $V_m$, was $2.33 \text{ Å}^3/$dalton.

(B) The same plot for about 3000 protein crystals whose data were taken from the CARB/NIST crystallization database. The mean value for $V_m$ is little changed from that first calculated. Although individual deviations are far greater, it is likely that some reflect errors. Both the original and current plots tend to suggest that the spreads broaden considerably for larger unit-cells. The plot in B was provided by J. Ladner.


The lower the solvent content the better the packing and, often but not always, the better the resolution.

Finally...there are many useful websites on protein crystallization, including:

http://www.hamptonresearch.com/products/
http://www.emeraldbiosystems.com/
http://alpha2.bmc.uu.se/terese/crystallization/library.html

And more books, like:


So Iwata, Ed. (2003) Methods and Results in Crystallization of Membrane Proteins (IUL Biotechnology)