Protein Crystallization, Crystal CryoProtection and Crystal Annealing

Of water soluble proteins only – but many principles apply to membrane proteins and protein-nucleotide complexes as well.

January 2011

Wim G.J. Hol
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 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)
**How to make the most of your precious protein solution?**

**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

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

**Limited Proteolysis**

Provides information about:
- 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
- Useful “chunks”
  - For crystallization after or without purification
  - For creation of “truncated constructs”

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

Tbru018012AAA, R5679

Tcru02395AAA, W8424

Key:
- **Time for proteolysis**
- $0 = 0$ hour
- $1 = $ Proteolysis in $1$ hour
- $24 = $ Proteolysis in $24$ hours

**4 Proteases:**
- $T = $ Trypsin
- $Th = $ Thermolysin
- $S = $ Subtilisin
- $G = $ Glu-C

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

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Worth considering

**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

For each protein there are in principle millions of conditions to be explored…

How to walk efficiently through protein crystallization space??
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...

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
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…

- Currently, it appears that medium Mw PEGs (3-4 KDa) plus 100 to 500 mM salt are possibly the most successful crystallization solutions for the average protein (of course, not for your proteins). A wide variety of salts are available in so-called “PEG-ion screens”.

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.

Top twelve from: Alex McPherson

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

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Table 5.4. Dielectric constants of organic solvents

<table>
<thead>
<tr>
<th>Name</th>
<th>Dielectric Constants</th>
</tr>
</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 sulfate</td>
<td>42.60</td>
</tr>
<tr>
<td>Glycerol</td>
<td>42.50</td>
</tr>
<tr>
<td>Nitromethane</td>
<td>39.40</td>
</tr>
<tr>
<td>Ethylene glycol</td>
<td>37.70</td>
</tr>
<tr>
<td>N,N-dimethyl formamide</td>
<td>37.50</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>

Table 5.1. Methods for attaining a solubility minimum

<table>
<thead>
<tr>
<th>Method Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Bulk crystallization</td>
</tr>
<tr>
<td>2. Batch method in vials</td>
</tr>
<tr>
<td>3. Evaporation</td>
</tr>
<tr>
<td>4. Bulk dialysis</td>
</tr>
<tr>
<td>5. Concentration dialysis</td>
</tr>
<tr>
<td>6. Microdialysis</td>
</tr>
<tr>
<td>7. Liquid bridge</td>
</tr>
<tr>
<td>8. Free interface diffusion</td>
</tr>
<tr>
<td>9. Vapor diffusion on plates (sitting drop)</td>
</tr>
<tr>
<td>10. Vapor diffusion in hanging drops</td>
</tr>
<tr>
<td>11. Sequential extraction</td>
</tr>
<tr>
<td>12. pH – induced crystallization</td>
</tr>
<tr>
<td>13. Temperature – induced crystallization</td>
</tr>
<tr>
<td>14. Crystallization by effector addition</td>
</tr>
</tbody>
</table>

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 0.1+0.1 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”
Vapor diffusion - Hanging drop variant

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.

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.

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.

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**Microbatch Under Oil Crystallization Screening in a 1536 Well Microassay Plate**

From: George DeTitta and co-workers – HWMRI, Buffalo.
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

1 = after adding the volumes to capillary
2 = after making layers touch – often by (mild) centrifugation
3 = after crystals grow - often by magic.
(Often NOT at interface)
A robust and scalable microfluidic metering method that allows protein crystal growth by free interface diffusion

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.)
(B) Loading of reagents using pressurized outgas priming method. The interface valve (Center) is actuated, and reagents are loaded into adjacent sides of compound wells. (Lower) Wells are being deadend-loaded with water. (Upper) Wells have been loaded with 13mM bromophenol blue sodium salt (Aldrich).

(C) A gradient of dye concentration. The containment valves (Upper and Lower) isolate compound wells, and the interface valve is released to allow diffusive mixing. The image shows complete mixing after 2 h. (Scale bars, 1 mm.)

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

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Walking through
Crystallization Space using
Phase Diagrams

Very Different
for
Different Crystallization Methods
**Vapor Diffusion**

Each Individual Well a Unique Vapor-Pressure End Point

**Classic Micro-Batch Under Oil**
(Non-permeable oil and trays assumed)

After the initial mixing step no changes.
**Modified Micro-batch Under Oil**

*(Water-permeable oil)*

Each well a fatal and dry End Point

**Free Interface Diffusion**

*(impermeable reservoir material)*

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

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**
Dialysis using “microdialysis buttons” (Zeppezauer)

Popular with electron microscopy experts to grow 2D crystals in the presence of lipids.


Altering the pH by diffusing in a volatile buffer though vapor phase

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

Ethanol

Protein solution

Reservoir with aminoethanol in ethanol-buffer mixture

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

From a paper by Alex McPherson in either Nature or The Scientific American, or both
Crystallization: a multi-step process

- **Step 1: Initial Screening**
  - Determine lead conditions for crystallization
  - Using a “coarse screen” also called “random screens” (there are many commercial so-called “sparse matrix random screens” available).

- **Step 2: Optimization of “hits”**
  - Optimize lead conditions to produce diffraction quality crystals by “optimization matrices around” initial hits, e.g. varying pH, precipitant concentration, in small steps around the initial hit.

Often, many optimization-generations needed

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Temperature Variation

A subtle way to obtain (much better) crystals
(Even 14 C can give different crystals than 20 C)
SEEDING
A splendid way to obtain (much better) crystals from 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 dilutions from the “solution with seeds”
  - **Macro-seeding** - “Clean and grow again” - partial dissolving of crystals to “clean the surface”, then increase protein concentration

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**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

B. Wash crystal repeatedly in stabilizing solutions

C. Transfer crystal to pre-equilibrated drop

Recent application:
The Fab fragments of 3 antibodies were crystallized in complex with the antigen human IL-13. The initial crystallization screening for each of the three complexes included 192 conditions. Only one hit was observed for H2L6. None were observed for the other two complexes. Matrix self-microseeding using these microcrystals yielded multiple hits under various conditions. These were further optimized to grow diffraction-quality H2L6 crystals. The same H2L6 seeds were also successfully used to promote crystallization of the other two complexes. The M1295 crystals appeared to be isomorphous to those of H2L6, whereas the C836 crystals were in a different crystal form.

ADDITIVES
Another way to obtain better crystals from 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”).

or:

- assist in forming crystal contacts (the so-called “glues”)

Sometimes the same small molecule can serve as “freezer” and as “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”
Specific Synthetic Ligands Can Change Crystals
(but in this remarkable case left cell dimensions the same!)

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*

(in providing complex ligands for a real good price)

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!

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**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
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.

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: Sitting drops
Acapella: Capillary Xtal growth
RoboDesign MicroScope II

Database and Image Archive

Larry DeSoto & SGPP
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
  - Expose different pieces of the same crystal - in particular on so-called “micro-focus” synchrotron beams
  - Soak in Heavy-Metal Compounds, even repeatedly
  - 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 approximately 0.25 mm.


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**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>
</tr>
<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>Xyliitol</td>
<td>22 (w/v)</td>
</tr>
<tr>
<td>(2R,3R)-(-)-butane-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 (MPD)</td>
<td>20-30 (v/v)</td>
</tr>
</tbody>
</table>

The list was compiled from unpublished observations in the labs of Stephen Harrison and Don Wiley, and a survey of structures and reports in six journals between 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:
FLASH ANNEALING

Glycerol Kinase Diffraction pattern - Prior to Flash Annealing

FLASH ANNEALING

...and after 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
- But sometimes…..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: 5 >> 2.7Å**

- 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:** 2.7Å, 55% solvent

DsbC-DsbD: 7 >> 2.3 Å

- 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


Dehydration - Rehydration

- Pyruvate Dehydrogenase Multi-enzyme complex:
- E2 component forms a highly symmetric core around which E1 and E3 components assemble

- Initial diffraction of Icosahedral E2 crystals: 7 Å from non-dehydrated drop, mosaicity=1.09°

- Rehydration method
  - Take crystals from completely dehydrated drops (28 months after set-up); resuspend in cryo buffer (ML + 30% glycerol) and cool 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: “Surface Entropy Reduction”
  – Make truncations or elongations
  – Try homologs from other species
  – Form complexes with partner proteins
• **With lots more work:**
  – Prepare antibodies – F\textsubscript{ab}’s or F\textprime;’s or “nanobodies”
    • Phage display for generating specific antibodies
    • Classical hybridoma techniques
**Nanobodies**

(a) Classical Ab  
(b) Heavy-chain Ab  
(c) VHH domain: 15 kDa monomeric prolate particle: 2.4 nm x 4 nm "nanobody"  
(d) Advantage: All affinity in one single domain  

**Production of antigen-specific nanobodies**

- Collect blood  
- Isolate lymphocytes  
- Extract mRNA  
- Select Ag-specific Nb’s by panning  
- Make library of ~10^7 transformants  
- Produce soluble antigen-specific Nb’s  
- Ship ready-to-use Nb’s to Seattle  

Nanobodies prepared by: Els Pardon, Jan Steyaert @ VUB&VIB, Brussels  

CDR = Complementarity Determining Region
The I:J:nb11 complex

Front view

Top view

<table>
<thead>
<tr>
<th>J:nb11 interface:</th>
<th>ASA, Å²</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDR1</td>
<td>121</td>
</tr>
<tr>
<td>CDR2</td>
<td>87</td>
</tr>
<tr>
<td>CDR3</td>
<td>478</td>
</tr>
<tr>
<td>Framework</td>
<td>45</td>
</tr>
<tr>
<td>Total nb</td>
<td>731</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TruncI:truncJ interface:</th>
<th>ASA, Å²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1450</td>
</tr>
</tbody>
</table>


I:J:nanobody crystals

Nanobodies are great for crystal packing

Crystals this time within days...

ETEC peri-D plus nanobodies

Tight heterotetramer - two tetramers in crystal form I & one in crystal form II

Konstantin Korotkov, Seattle & Els Pardon, Jan Steyaert, Brussels

2.1 Å resolution for A6:Nb15

Years of no crystals despite immense efforts. With nanobodies crystals within two weeks.

Meiting Wu, Young-jun Park, Stewart Turley, Els Pardon, Jan Steyaert - J Struct Biol, in press
Crystal packing of A6:Nb15

SAME packing in the A6:Nb5 crystals
While Nb5 and Nb15 differ ~ 25% in sequence...

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

Usually when crystals are ill-suited for understanding mechanisms or for “iterative structure-based drug design” or for “fragment cocktail crystallography”

Basic Idea is: Change the crystal contacts in the current crystals.

---

**Plasmodium falciparum**

Peptide Deformylase (PfPDF)

various crystal forms obtained by contact residue alteration

<table>
<thead>
<tr>
<th>Protein Variant</th>
<th>Inhibitor</th>
<th>Space group</th>
<th>Cell dimensions (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>none</td>
<td>P4₁</td>
<td>a=b=121.3  c= 177.3</td>
</tr>
<tr>
<td>Engineered “393”</td>
<td>“393”</td>
<td>P6₅</td>
<td>a=b=102.4  c= 118.3</td>
</tr>
<tr>
<td>Engineered 944</td>
<td>944</td>
<td>P3₁21</td>
<td>a=b=65.1   c = 82.7</td>
</tr>
<tr>
<td>Engineered 11T</td>
<td>11T</td>
<td>P4₁</td>
<td>a=b=76.0   c = 155.1</td>
</tr>
</tbody>
</table>
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
http://ffas.burnham.org/XtalPred-cgi/xtal.pl

And books, like:
Alex Mcpherson (1985) “Crystallization of Biological Macromolecules”


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

Good to read:

“Turning protein crystallisation from an art into a science”
Naomi E Chayen
Current Opinion in Structural Biology (2004) 14:577–583

Crystal Growth Chapter in the book:
“Biomolecular Crystallography: Principles, Practice, and Application to Structural Biology”
Bernard Rupp (2010)
Older Literature

Methods in Enzymology, Vol 114
"Diffraction Methods of Biological Macromolecules"

Methods in Enzymology Vol 276
Section II "Crystals"-Carter and Sweet(1997)
Macromolecular Crystallography Part A