

## **L -2    Sample preparation**

**Before crystallization (first step-most important)**

### **Points to consider (check list)**

- 1) Sequence**
- 2) Stability**
- 3) Solubility**
- 4) Purity**
- 5) Quantity**

## 1) Sequence (amino acid composition)

**Flexible:**                      **Glycine**

**Hydrophobic:**                **Alanine, Valine, Phenylalanine, Proline,  
Methionine, Isoleucine, Leucine**

**Cysteine:**                      **Disulfide bridges**

## 2) Stability (conditions/time)

**Find a stabilization buffer.**

**Ideal buffer water, let the protein buffer itself.  
Rare to live in ideal world.**

**Use the **minimum amounts** (buffers, salts etc).**

**The protein has to be stable in the crystallization drop  
until it forms crystals. Ideal goal 2 weeks.**

**Stability is effected by the environment.**

### **3) Solubility**

**The more soluble the protein the more likely it is to crystallize.**

**Myoglobin 1955 (Kendrew)**

**Lsozyme (Phillips)**

**Hemoglobin (Perutz)**

**Real world 10mg/ml**

**And then membrane proteins.**

## 4) Purity

**Nothing can be too pure!!!!**

**95% try**

**99% really what it takes**

**Detection: SDS gel electrophoresis (silver stain)  
Don't run sample off the gel.**

## 5) Quantity

**Crystallography can be soul destroying.**

**Need a concentration of 10mg/ml.**

**A trained person:**

**A single condition (DROP) will require a minimum of 2 $\mu$ l (5 $\mu$ l) of sample.**

**Hence 100 conditions (ONLY DONE ONCE) will use up 0.2 ml of sample (2mg). (1mg = 50 conditions)**

**Robot:**

**Requires 0.6 ml (6 mg) to test 96 X 16 conditions 1536 (Person 3.1 ml 30.7 mg).**

## **Lyophilization:**

**Avoid lyophilization of your protein for storage if you plan to use the protein for crystallization experiments. Also don't concentrate it.**

**Lyophilization is a not so gentle procedure and can prevent crystallization. **So don't go there.****

**If you have a lyophilized protein **is there hope ?****

**Should solubilize the protein in water or a stabilization buffer **dialyze the protein exhaustively.****

**Dialysis is an important step which will help to remove residual, non-volatile buffers and reagents as well as low molecular contaminants.**

## **Sample aggregation:**

### **Deterrent to crystallization**

**Detection:**                    **Dynamic light scattering**  
                                      **Native gel electrophoresis (silver stain)**

**Common Causes:**        **Hydrophobic patches on the surface**  
                                      **Differently charged isoforms**  
                                      **Differently phosphorylated isoforms**  
                                      **Mixtures of methylated and**  
                                      **non-methylated samples**  
                                      **Glycosylation**  
                                      **Electrostatic interactions**

## Two types of aggregation

**Heterologous** contamination where the sample is aggregating with other proteins

**In the case of heterologous contamination, further purification of the sample should be seriously considered.**

**Autologous** aggregation where the protein is aggregating with itself

**Remedies:**

**Molecular biology:**

**Manipulate intra and inter molecule interactions by modifying the sample sequence (alter, add, or delete residues). Consider a fusion protein. Remove C-terminus or N-terminus. Truncate domains. Remove His-tag**

**Chemical additives:**

**Manipulate sample-sample and sample solvent interactions.**

**Detergents**

**Alcohols (isopropanol, methanol, ethanol, etc)**

**Salts (sodium chloride, potassium chloride, sodium fluoride, etc)**

**Polyols (glycerol, PEG 400, etc)**

**Ligands, inhibitors, co-factors, and metals**

## **Other suggestions:**

**Use temperature to prevent aggregation (0°C and 60°C)**

**Centrifugation or filtration.**

## **Last hope:**

**Mixing the sample with the crystallization reagent, allowing the sample to incubate for 15 minutes, centrifuging the sample/reagent mixture, removing the precipitate and setting the drop with the supernatant.**

## **Proteases:**

**Proteases can also be trouble, causing cleavage of the sample.  
(Talk to Prof. Ben Dunn)**

## **Remedy:**

**Add a protease inhibitor.**

**Protease contamination can be carried over from isolation and purification of the sample.**

### **Protease: Metalloproteases**

**Inhibitor: Chelators like EDTA and EGTA, bestatin, amastatin, thiol derivatives, hydroxamic acid, phosphoramidon.**

### **Protease: Aspartic Acid Proteases**

**Inhibitor: Pepstatins and statin derived inhibitors**

### **Protease: Cysteine Proteases**

**Inhibitor: Thiol binding reagents, peptidyl diazomethanes, epoxysuccinyl peptides, cystatins, peptidyl chloromethanes.**

### **Protease: Most**

**Inhibitor: DEPC**

### **Protease: Serine Proteases**

**Inhibitor: Trypsin inhibitors, leupeptin, boronic acids, cyclic peptides, DIFP, PMSF, Pefabloc, aminobenzamidine, 3,4-dichloro isocoumarin, chymostatin.**

**The presence of fungal or microbial contamination in your sample or reagents, with subsequent release of proteases from these organisms.**

### **Remedy:**

**Include sodium azide or thymol**

**Deterrent to microbial growth, which prevents the possibility of microbial agents growing and secreting proteases into the sample solution. Typically add 1mM sodium azide.**

### **Note:**

**Sodium azide and thymol can sometimes bind the sample, are toxic and in some cases do not live well with heavy atoms**

### **Our Lab:**

**Rather, clean workspace, sterile filtered samples and reagents good technique prevents microbial contamination.**

**However:**

**Proteolytic modification of proteins can be a tool for making small active fragments of proteins, which might have enhanced solubility characteristics compared to the native protein, which in turn might make the protein more amenable to crystallization.**

## **Nucleases:**

**Problems:** Modification of size, charge or hydrophobicity, partial or total loss of activity, or utter destruction of nucleic acids.

**Difficult to detect even on overloaded electrophoresis gels.**

**Damage can be during purification, concentration, and storage.**

**Remedies:** Include a nuclease inhibitor in the prep or sample to protect the sample from ribonuclease and deoxyribonucleases.

**Inhibitors:** RNasin (from Promega), ribonucleoside-vanadyl complexes, and DEPC. Inhibitors of deoxyribonucleases include DEPC and chelators such as EDTA or EGTA.

## **Reducing Agents:**

**Oxidation can lead to non-specific aggregation, heterogeneity, inactivity, or denaturation of protein.**

## **Remedy:**

**Reducing agents are substances that cause other chemical species to be reduced or gain electrons. In order for reducing agents to cause the gaining of electrons on some other chemical species they must undergo oxidation and prevent the oxidation of free sulfhydryl residues (cysteines) in proteins. (Also glove box)**

## **Examples:**

**Dithiothreitol (DTT), beta-mercaptoethanol (beta-me), and Tris(2-Carboxyethyl)-Phosphine Hydrochloride (TCEP-HCl)**

**Concentration range of 1 to 10 mM in the crystallization drop.**

## **Crystallisation improvements of samples (examples)**

### **Site directed mutagenesis:**

**Recombinant DNA technology-means to make specific changes in a protein sequence**

**-Systematic approach of improving crystallisability by changing hydrophobic to hydrophilic residue:**

**e.g. HIV integrase**

**(Davies, Science 1994, 255: 1981-1986)**

**-Alterations of protein and RNA sequence to obtain a more stable complex:**

**e.g. U1A protein-RNA complex**

**Outbridge, JMB 1995, 249:409-423)**

## **Fusion proteins:**

**Carrier protein introduced into an internal position of a target protein**

**-Carrier protein, soluble, single compact domain, crystallisable, N- and C- close together, no disulphide bonds, easily cloned and expressed, larger than target protein**

**e.g. Introduction of the *E.coli* Cytochrome b562 into lactose permease (membrane protein)  
(Prive, Acta Cryst. D. 1994, 50:375-379)**

## **Complexes with antibodies:**

**-Proteins of interest are complexed with Fab fragments  
(Concept same as fusion proteins)  
e.g. P24:Fab complex (Kovari, Structure 1995,3:1291-1293)**

## **Odd result:**

**-Improvement of crystal lattice contacts by mutations:  
e.g. Glutathione reductase E.coli  
(Schulz, Acta Cryst. D. 1994, 50: 228-231)  
Double mutation >>Crystals grew 40 times faster  
than for native protein**

## **Finally questions to ponder about YOUR sample:**

- **Does a similar sample exist and has it been crystallized?**
- **Does the sample contain free cysteines?**
- **Does the sample contain additives such as sodium azide, ligands, inhibitors, or substrates?**
- **Is the protein glycosylated?**
- **Is the protein phosphorylated?**
- **Is the protein N-terminal methylated?**
- **At what temperature is the protein stable?**
- **How does sample solubility and stability change with temperature?**
- **How does sample solubility and stability change with pH?**
- **Does the sample bind metals?**
- **Is the protein sensitive to proteolysis?**
- **What class of protein/NA am I working with (antibody, virus, enzyme, membrane protein)?**

- **What has been the most successful approaches with my class of protein?**
- **What is the source of the sample?**
- **How was the sample purified and stored before it arrived into my hands?**
- **What is in the sample besides the sample (buffer, additives, etc)?**
- **Is the sample pooled purification aliquots or a single batch?**
- **How much sample do I have and how much more is available?**
- **How pure is the sample?**
- **How homogeneous is the sample?**
- **Does anyone possess any solubility information on this sample?**
- **What is unique about this protein?**
- **What is necessary chemically and physically to maintain a stable active sample?**

## **Last thought:**

**“Never, never, never see protein purification as a separate event from crystallization.”**

**It should be seen as a continuous process.**

**Crystallization is the final step in purification.**

## **Assignment:**

**In half a page (note form)**

**Think about the questions above, and write a profile that might help you crystallized YOUR sample**