PREFACE

Protein Crystallization and Dumb Luck

I hope I am not shooting myself in the foot writing an article using the words protein crystallization and dumb in the title. I am not out to ruin crystallography as the weapon of choice for solving the three dimensional structures of the human genome proteins. Nor am I out to scare those of us who have grant applications in with a big fat emphasis on protein crystallization and crystallography. But dog gone it, I am growing tired of this age of political correctness. It is time to say it like it is. A lot of protein crystallization is dumb luck. There, I said it; it's out there. Why my penchant for dumb luck. Perhaps it is because my graduate advisor used to tell me "Cudney, I don't know how you get these crystals, it must be a lot of dumb luck". What ever works, right?

Well, sure a lot of it is well thought out, elegant science in protein crystallization, but lots of turn and burn crystallization stuff is dumb luck. How do we know this? We do not read about dumb luck. Well, maybe we do, but it has been disguised as science. However, obvious dumb luck is not something we like to publish. We might back up and wrap the dumb luck in an elegant idea, but I have not yet read a Material and Methods Method section yet referencing dumb luck as the ingredient in any crystallization trial. But dumb luck is something we hear about at poster sessions, coffee breaks, in the vendor booths at trade shows, and while we are waiting for crystals to grow. We all experience dumb luck, whether we want to admit it or not. Think about it. How many times have you purposely designed a crystallization experiment and had it work the first time? Liar. Like you really sit down and say "I am going to use pH 6 buffer because the p1 of my protein is just above 6 and I will use isopropanol to manipulate the dielectric constant of the bulk solvent, and add a little BOG to mask the hydrophobic interactions between sample molecules, and a little glycerol to help stabilize the sample, a pinch of trimethylamine hydrochloride to perturb water structure, and finally add some tartate to stabilize the salt bridges in my sample". Right. We try what has worked for us or others before and that is how we grow protein crystals. Okay you say, we can get crystals from a first run screen, explain that. Easy, there is dumb luck built into these screens so that doesn't count. Okay, so I am trying to be funny here, but you almost have to admit, there is a little dumb luck in every crystallization. Yes, let's hope the big guns on the panels for the human genome project and structural biology related grants skip this article or have a great sense of humor. But we have to face it. Finding the best crystallization conditions is a lot like looking for your car keys; they're always in the last place you look.

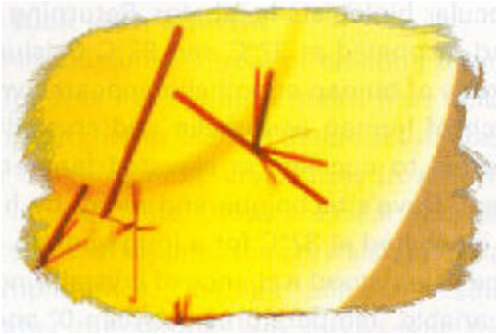
The first record of dumb luck protein crystallization

It has been said that Professor Hunfeld sacrificed a lowly earthworm for a drop of blood and placed this drop of blood along with perhaps a bit of buffer solution onto a glass microscope slide. Professor Hunfeld then sandwiched the drop of blood with a cover glass without sealing the edges and proceeded to have a look at the blood under a microscope. It is then said that Professor Hunfeld observed the appearance of red solid bodies in this liquid specimen, and declared the solid bodies blood crystals. One would assume that perhaps Professor Hunfeld was actually looking at the first crystals of hemoglobin. Here, Professor

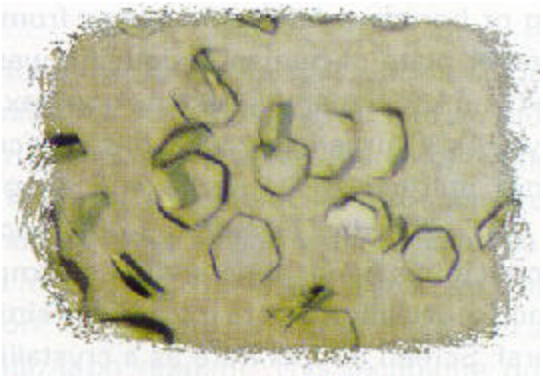
Hunfeld, without ever intentionally trying to crystallize a protein, demonstrated that it was possible to crystallize biological macromolecules. Second, he demonstrated that utilizing evaporative methods to control the relative supersaturation of the sample in the set up could grow protein crystals. Over one hundred years later most of us still use crystallization methods based upon Professor Hunfeld's "accidental" discovery in 1840. That's powerful dumb luck.

Dumb luck and human endothelin

In 1989 I was working with a fledging pharma wanna be. We had a crystallographer, an idea, and were on the hunt for millions of dollars and a public offering. Business development by day,crystal growth and map fitting by night. This biotech preemie had all the dreams, some of the talent, none of the money, and drug targets that seem regulated by Murphy's law. The hope was to make human endothelin antagonists that would some day be used to regulate hypertension.At this time human endothelin was a relatively new research target, a 21 residue peptide that could be synthesized by a talented peptide chemist. Human endothelin was not inexpensive. At that time it cost more than $2,000 per milligram. The company bought 25 milligrams. The crystallography group was supplied with a whopping 5 milligrams with the anticipation of successful crystallization and subsequent structure determination by X-ray diffraction analysis. At this point in history sparse matrix crystallization screens were an underground phenomena that were scoffed at by many crystallization experts and were not yet available commercially. Hence,we were left with homemade screens. Our lab was equipped with a homemade vintage version of the Jancarik and Kim screen (which would eventually become Crystal Screen). We were also fans of rudimentary grid screens. Grid screens are simple yet effective screens that screen single precipitants such as salts, polymers, and organic solvents versus a range of pH such as 4 to 9. Once the human endothelin sample arrived the sample was solubilized in water to 15mg/ml and set up against the sparse matrix screen consuming 48 microliters of our allotted 300 microliters. After a few days, spires of hexagonal plates were observed in drops containing MPD at a neutral pH. Next we set a grid screen based upon MPD versus an appropriate pH range. Voila! Once again we observed the spires of hexagonal plates but were now blessed with a few hexagonal rods. The rods gave us hope that we might get something large enough to put onto the area detector. Recall we are in the age where cryocrystallography was just a twinkling in a few researchers' eyes and a synchrotron trip was not yet as convenient as fast food. With some further refinement of the preliminary crystallization conditions, altering MPD concentration and pH we were able to push the crystals to something large enough for a capillary. For you whippersnapper variety crystallographers we used to suck (literally) crystals into capillaries made from glass. Then we would carefully position and seal the crystal into the capillary with just the right amount of mother liquor (protein and reagent), wax, and glue (very high tech). Finally, we would then watch the crystal (a) dissolve (b) crack (c) slide out of position or (d) be destroyed by the X-ray beam. In any event, low-resolution diffraction was achieved, proving we did indeed have the first crystals of human endothelin. Attempts at using the low-resolution data with direct methods failed (low resolution; direct methods -- is this an oxymoron or what?). The company, not completely willing to bet its life on crystallography also employed and touted 1980's big hair molecular modeling. Yes, we too had a multi-processor over priced, "slower than a 1999 DelI home PC" supercomputer with in house patented software that could generate perfect structures from an amino acid sequence. Well, surprise surprise, using molecular replacement we were not able to generate a structure from the model coordinates. The management challenged our X-ray data. It could not possibly be an error with the computer generated model, we had paid too much for that. Additional crystals were required for heavy atom derivatives to save our jobs. The tech, which we will call Dave, who set the original crystals, went on holiday. Crystallization set ups were performed in Dave's absence. No crystals appeared. Using the same solutions, same pipettes, and the same sitting drop method, no crystals were obtained. Upon his return, the tech repeated the previously successful conditions and obtained crystals. Now folks,this is a



Hemoglobin



Edestin

Ribonuclease A

typical example of why crystal growth has been labeled a “black art”. Undaunted, we set out to see what might be the difference in Dave’s set up and those who were unable to reproduce the crystals of endothelin. I sat directly beside the tech and together we performed crystallization set-ups with human endothelin using the same method, the same reagents, and the same sample. We had two innocent molecular biologists that had never set crystallization trials watch our progress during the set-ups. In the end, the only difference noted between my technique and that of Dave’s was that I was anal enough to leave my sample chilled in the ice bucket during drop set up while Dave held the sample in his nice warm hand. The next day we observed no crystals in my set up while the tech enjoyed tremendous success and many crystals. Perhaps the most ironic part of this scenario is that this was Dave’s first exploration into protein crystallization, while I on the other hand was a survivor of an illustrious crystallization lab. In the end, we sought the solution of our plight through a crystallization Jedi Master. “Try temperature” he said. “We did that” we replied. “What temperatures did you try” the master queried. “4°C and room temperature” we cockily snapped, as does most everyone else who routinely screens temperature. When asked if we had tried any temperatures above room temperature, we stupidly replied “Huh?” Being typically practical with loads of common sense and a high appreciation of low budget methods, the master suggested we find a warm room in the building or borrow a few cubic inches from a molecular biologists incubator. Returning to the lab, another plate of human endothelin was set and incubated at 37°C and 57°C. And wouldn’t you know that the best looking hexagonal rods of human endothelin appeared within a few days. As it turned out, with our particular batch of human endothelin and crystallization conditions utilizing MPD, the hexagonal rods preferred to nucleate at elevated temperatures around 37°C. Thinking back to the difference between Dave’s technique and mine, by holding the sample in his hand, apparently the sample was incubated at

37°C for a long enough period to promote nucleation where my sample sitting on ice never stood a chance of crystallizing.

Moral: Screen temperature as a crystallization variable. Temperatures between 0° and 60°C should be considered. Some additional tidbits. The effects of temperature are typically amplified in low ionic strength. While most of us favor hanging drop vapor diffusion as the method of choice realize that: 1) Condensation can be a problem when vapor diffusion experiments experience a decrease in temperature. With a hanging drop experiment the water droplets condense on the glass slide and can coalesce and dilute the sample droplet. This is less likely to occur with a sitting drop vapor diffusion experiment. 2) Condensation can interfere with the viewing of a hanging drop vapor diffusion experiment. So what. Simply remove and replace the tape or cover slide. 3) Thermal stability of the drop. Best to worst. Microbatch > Sitting drop on glass rods > Sitting drop on glass slides or plastic supports > Hanging drop vapor diffusion.

Dumb luck and ribonuclease A

While up setting crystals of bovine pancreas ribonuclease A, I faced another challenge with temperature. Using the ICN Crystal Plate (plastic framework with glass slides that allows for hanging, sitting or sandwich drop), we carefully set the plate in a large, glass cake dish full of water in an attempt to buffer the anticipate temperature increase from leaving the plate under a light microscope for a few days while the crystals grew. Crystals grew nicely in the control experiment before being loaded onto the microscope and high tech cake dish thermal buffer. But crystals were bashful and would not appear under the microscope even after several days of equilibration. The crystals would always appear overnight after the video taping had stopped and everything was shut down. What was going on? Once again, a temperature effect. It was noted that crystals that appeared overnight, with the light microscope turned off, could be dissolved by turning on the microscope light and leaving the plate in the bath for several hours.Upon measuring the temperature of the water bath before and after leaving the light microscope illuminated for several hours it was determined that the temperature change betweenlights on and lights off was “subtle”, only several degrees Celsius. In fact, later we were able to nucleate and grow crystals at one temperature, then by elevating the temperature etch back larger crystals and dissolve smaller crystals. Then, lowering the temperature, grow the remaining crystals larger.

Moral of the story: “subtle changes in temperature can be significant in the nucleation and growth of protein crystals. Not only is it important to screen a variety of temperatures but it is important to be aware of the effects that temperature fluctuations might have on the nucleation, growth or dissolution of the crystal. Finally, try temperature cycling for nucleating and growing crystals. This can be done on a high budget with multiple incubators or on a low budget by finding cool and warm places to hide plates. To conserve sample, one can simply move old plates to different location with different temperatures and observe drops for solubility changes. Phase changes (clear to precipitate, precipitate to clear, precipitate to crystals, etc.) due to temperature change is a pretty obvious clue that one should incorporate temperature into the optimization regime.

Dumb luck with edestin

While pursuing a goal of creating a large portfolio of protein crystallization standards by screening lots of exciting proteins from scientific mail order catalogs we ran into an old friend edestin from hemp seeds. Edestin solubilizes into tan/gray slurry with water and following filtration or centrifugation, crystallizes in a number of different conditions. All producing crystals buried in precipitate. Drops are clear upon set up and then after a short while precipitate forms, then a short while later the crystals appear as gems in the slag. It was suggested we centrifuge the sample twice. One spin following “solubilization” with water and a

second spin a short while after the sample was mixed with the crystallization reagent. This technique produced fewer and larger crystals of edestin without the appearance of amorphous precipitate.

Moral of the story: If precipitate is interfering with your crystallization trials after set up, try centrifuging the samples after mixing with crystallization reagent, then set the drops. This might also be a long shot worth trying when one suspects the presence of non-specific aggregates forming in the sample and interfering with the crystallization.

Dumb luck with lactate dehydrogenase

Recently we have been working with lactate dehydrogenase from chicken muscle. This stuff is easy to crystallize from a number of screens but does not readily form large single crystals, preferring to form expressive needles and anemic plates. Our most energetic protein people added a chromatography clean up step to the prep to see if this might be a quick detour around the needles and plates. Upon receipt of the sample, lunch was selected over pipetting and the sample was stored at 4°C. Upon return from lunch it was noted the previously clear sample had turned white with turbidity and there was quite a bit of "precipitate" on the bottom of the tube. Gently rocking the sample it was noted the precipitate displayed a nice sheen in solution rather than the typical lumpy dead precipitate. Upon closer inspection of a few microliters of the sample on a glass slide it was duly noted the thoroughly dialyzed sample had undergone crystallization when the sample was chilled at 4°C.

Moral of the story: Look closely at your precipitates for "thar may be crystals in them hills of precipitate".

Dumb luck with snake venom and a breast cancer Fab

In an effort to add life to the sleepy protein standard portfolio we somehow landed our hands on a snake venom protein and a Fab generated to a breast cancer protein. Vagueness is intentional here to protect the pharma giants who supplied the proteins. Repeated screens with both proteins resulted in lots of clear drops and lots of phase separation and lots of precipitate.Nary a single crystal. Living under the credence "Never throw away a plate until the reservoir is a dry crust" we rediscovered the plates following a move of the lab. The reservoirs had magically decreased to one half the original volume over the course of a year or so and in a few drops, the stubborn snake venom and breast cancer Fab had lazily formed crystals. What might have happened here? Well, one might say that the protein had undergone proteolytic or chemical modification during the storage. Or one might argue that evaporation of water from the reservoir led to an increased level of supersaturation in the drop, finally forcing the protein into crystalline form. And there is no telling how much temperature cycling and vibration had to do with this phenomenon. Suffice it to say, keep your plates as long as you can and keep an eye on them so you can have a better idea of what caused the kind event of crystal growth. In our lab we take a peek at plates immediately after set up, a day later, and once a day for the first week.Then once a week until the drop dries to a dead crust. And be sure to check that crust is not crystalline protein.

Dumb luck and polymers as crystallization reagents

We all know that in many instances polyethylene glycols work wonderfully as crystallization reagents. Perhaps many of us also know the merits of other novel, yet less frequently utilized polymers such as Jeffamine and the polyethylene glycol monomethyl ethers.

Upon return from a scientific road trip to Europe and Russia our crystallization mentor was enlightened with the many chemical virtues of polymers and how they can affect the structure of water. Our mentor was in turn enlightening us with loads of chemical trivia which might explain how these polymers affect water structure and how one might expect it to be reasonable to explore a variety of water soluble polymers as

crystallization reagents. But what convinced us to undertake this menial task was the following. Certain water-soluble polymers can be applied to the bottom of boat hulls to make them faster in the water. Some fire authorities add special polymers to municipal water, which allows larger volumes of polymer- treated water to pass through a fire hose compared to untreated water. Armed with nothing more than this bit of trivia and no specific list of polymers we did the next reasonable thing, ordered samples of every polymer we could find in the mail order chemical catalogs. Upon arrival, attempts were made to formulate the polymers into water at the highest possible supersaturation. The key word being attempt since the manufacturers did not detail the water solubility properties of many of these polymers. During the course of formulation we are certain we observed these polymers in unnatural states never imagine by the chemists who designed the polymers. In the end we settled on nine water soluble, pH neutral polymers and formulated them into a crude screen. All of the polymers produced crystals of some of the proteins tested, and of the 24 molecules (proteins,viruses, and small molecules) tested, 14 were crystallized. It was concluded that the potential range of such polymers useful in crystallization might be quite broad. We currently utilize the novel polymers in crystallization screens when it is suspected that polymeric chemicals may be an appropriate crystallization reagent. Hmm, nine new crystallization reagents that double as hull coatings for speedboats. Dumb luck strikes again.

Moral of the story: While is it wise to use common sense and perhaps follow statistical trends in the selection and representation of reagents in your crystallization screens, break out of your local minima reagent rut once in a while and try something new. A good screen will incorporate a proper balance of tried and true reagents, but also incorporate out liars to test dumb, er new ideas.

There are of course, more dumb luck stories, but perhaps we have all suffered enough.

So are we to be left to assume that the future of protein crystallization is in the hands of dumb luck? I think not. Dumb luck is an old weathered lab tool that has provided crystal growers with many interesting results. Results that were utilized to generate “real” experiments. Experiments which in turn generated data that have led to the synthesis of factual generalizations that we can use, together with common sense and scientific know how as powerful crystallization tools.

Over the past decade we have seen an exponential growth in the number of protein crystallization reports and subsequent structure determinations by X-ray diffraction methods. This enjoyable success has been fueled by an ever-expanding knowledge base of variables that can influence the nucleation, growth, and cessation of biological macromolecular crystals. Additionally we continue to learn new ways of how we can best manipulate and control these variables to obtain single crystals that diffract to high resolution. We now have access to a treasure chest of variables, screens, reagents, methods, and tools to crystallize biological macromolecules. The value of this treasure becomes more valuable with time as we learn how best to exploit and control these variables in order to obtain high quality single crystal for X-ray diffraction analysis.

It would be nice to think that all of our crystallization tools were rationally designed, like the next generation of pharmaceutical compounds. But in reality, perhaps a few of the today’s powerful crystallization tools were spawned from good old dumb luck. Anyone have any willow treebark? I think I feel a headache coming on.

May dumb luck be with you

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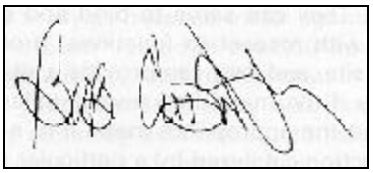

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