Advanced methods in macromolecular crystallization V

FEBS combined practical and lecture course PC12 023

Academic and University Center, Nove Hrady, June 22 - 29, 2012

Lectures - Saturday, June 23

L1

PROTEIN CRYSTALS AND THE PHYSICAL CHEMISTRY OF THEIR FORMATION

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Protein crystallization is a complex process that is a reversible equilibrium phenomenon involving three stages: nucleation, crystal growth and cessation of growth. The chemical and physical properties of the solvent and solute interplay are affected by specific kinetic and thermodynamic parameters. Both crystal nucleation and growth occur in supersaturated solutions such that the concentration of protein molecules surpasses its equilibrium value. Under selected conditions, the system is driven towards equilibrium state and consequently the protein molecules are partitioned between a soluble and solid phase through free energy minimization. Post nucleation crystallization events may proceed by mechanism associated with crystal defects or imperfection. Growth cessation of protein crystals is well observed but its processes remains unclear. The general principles of protein crystallization will be outlined concerning the behavior of macromolecules in solution and its fundamental aspects of the crystallization process will be reviewed.

L2

CONVENTIONAL CRYSTALLIZATION METHODS AND THEIR MODIFICATIONS

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With the phase diagram in mind, some of the shortcomings of conventional / common crystallization vapor diffusion set-ups will be discussed and alternative methods / modifications will be reviewed.

Classical vapour diffusion experiments can be modified by several pre and post set-up techniques:

A selection of pre set-up, vapour diffusion experiment alterations

1. insertion of an oil barrier that will slow down the equilibration rate
2. use of gels to, among other effects, slow down con vection in the droplet
3. use of capillaries in vapor diffusion mode to minimize handling of crystals
4. microseed matrix seeding to outwit nucleation
5. fluorescent dye-labelling of proteins
6. addition of proteases for *in situ*, limited proteolysis
7. One-for-all reservoir solution

A selection of post set-up, vapour diffusion experiment alterations

1. change reservoir precipitant concentration
2. change temperature
3. change pH
4. microseeding

Also the standard microbatch trials in Terasaki plates (droplets under paraffin oil) can be manipulated by choosing different oil (mixture of silicon and paraffin oil).

Most important, the experimentation does not stop after the cover slide is placed over the reservoir, so to speak.

Primers and Links

I. J. Drenth, Principles of Protein X-ray Crystallography (Third Edition, Chapter 16), Springer Science+Business Media LLC

II. T.M. Bergfors, *Protein crystallization strategies, techniques, and tips*, IUL Biotechnology series

III. A. Ducruix and R. Giegé, *Crystallization of nucleic acids and proteins*, Oxford University Press

IV. A. McPherson, *Crystallization of biological macromo lecules,* Cold Spring Harbor Laboratory Press

V. S. Iwata, *methods and results in crystallization of membrane proteins,* International University Line Biotechnology series

VI. N. Chayen, *Protein Crystallization Strategies for Structural Genomics*, IUL Biotechnology Series

VII. www.iobcr.org

L3

CRYSTALLIZATION OF MEMBRANE PROTEINS IN LIPIDIC MESOPHASES

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One of the primary impasses on the route that eventually leads to membrane protein structure through to activity and function is found at the crystal production stage. Diffraction quality crystals, with which an atomic resolution structure is sought, are particularly difficult to prepare currently when a membrane source is used. The reason for this lies partly in our limited ability to manipulate proteins with hydrophobic/amphipathic surfaces that are usually enveloped with membrane lipid. More often than not, the protein gets trapped as an intractable aggregate in its watery course from membrane to crystal. As a result, access to the structure and thus function of tens of thousands of membrane proteins is limited. The health consequences of this are great given the role membrane proteins play in disease; blindness and cystic fibrosis are examples. In contrast, a veritable cornucopia of soluble proteins have offered up their structure and valuable insight into function, reflecting the relative ease with which they are crystallized. There exists therefore a pressing need for new ways of producing crystals of membrane proteins. In this presentation, I will review the field of membrane protein crystallogenesis with reference to the latest update of the Membrane Protein Data Bank (www.mpdb.tcd.ie).

Emphasis will be placed on crystallization approaches which make use of the lipidic systems. In my talk I will describe these methods and our progress in understanding how they work at a molecular level. The practicalities of implementing these methods in low- and high-throughput modes will be examined. A practical demonstration of the lipidic cubic phase or *in meso* method will be given at the *FEBS Lab Excercises* on Saturday, June 23.

Useful References

Caffrey, M., Cherezov, V. 2009. Crystallizing Membrane Proteins In Lipidic Mesophases. Nature Protocols. 4:706-731. (PMID: 19390528)

Caffrey, M., Porter, C. 2010. Crystallizing membrane proteins for structure determination using lipidic mesophases. J. Vis. Exp. 45: www.jove.com/index/details.stp?id=1712 (doi: 10.3791/1712)

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L4

CRYSTALLIZATION OF PROTEIN-LIPID COMPLEXES OF THE IMMUNE SYSTEM

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The immune system developed a multitude of strategies to respond to infection, cancer, as well as control autoimmune responses. While antibodies can detect antigens directly in solution, T cells only recognize antigen when presented by antigen-presenting cells. The antigens are derived from microbes or viruses that have been phagocytosed and degraded in the antigen-presenting cells. Generally, short microbial peptides are then bound by antigen-presenting molecules (MHC I and II), which translocate to the cell surface for antigen recognition by T cells. The T cell that recognizes both the antigen-presenting molecule and the peptide becomes activated and proliferates to produce more T cells with the same specificity to fight infection. While the antigen-presenting cell can be directly killed by some cytotoxic T cells, other immune cells are also activated to limit spread of the infection.

Over the past decade, a parallel antigen-presentation system has been identified that responds to lipids and glycolipids, rather than peptides. The antigen-presenting molecule CD1 is structurally similar to peptide-presenting MHC but has a hydrophobic binding pocket suitable for the binding of various lipid backbones. The carbohydrate moiety of the glycolipid is presented above the opening of the hydrophobic pocket and acts as the major epitope for the T cell receptor (TCR) that is expressed on the surface of the T cell.

Our lab are interested into the recognition event, especially the crystal structures of the ternary complexes containing CD1-glycolipid-TCR. Structural characterization of the interaction allowed for the design of other glycolipids that lead to improved anti-microbial potency of those T cells by increasing the amount of secreted effector molecules (cytokines) that are necessary to activate other immune cells, such as Natural Killer cells. This has been useful in animal models of cancer, in which injection of these glycolipids leads to regression of the tumor.

The most widely studied population of T cells in both human and mice are termed Natural Killer (NK) T cells, as they express cell surface molecules that are characteristic of both NK and T cells. NKT cells broadly fall in two classes, based on their antigen specificity and their TCR usage. Type I, or V 14*i* NKT cells are evolutionary conserved and characterized by their V 14J 18 re-arrangement, as well as their reactivity to the prototypical glycosphingolipid -galactosylceramide (-GalCer). Type I NKT cells produce both pro- and anti-inflammatory cytokines (IFN- , IL-4, IL-17, TNF-) within 2-4 hours after antigen activation. They play a role in host defense against microbial pathogens, and contribute to the clearance of various cancers, due to their transactivation of NK cells leading to increased IFN- levels.

Type II NKT cells on the other hand are more diverse in their TCR repertoire, are not reactive to -GalCer and also do to have a common antigen. However, they also produce both pro- and anti-inflammatory cytokines within hours after activation. Among the Type II NKT cells, sulfatides are the best-studied antigens and sulfatide-reactive Type II NKT cells have a protective role in EAE, a mouse model for multiple sclerosis.

Using biophysical, biochemical and structural methods we have elucidate the mechanism of glycolipid-recognition by Natural Killer T (NKT) cells, which is the most abundant, lipid-reactive T cell population in mice.

We have determined several crystal structures for the Type I NKT TCR in complex with different, CD1d-presented glycolipids, such as -GalCer and its structural analogs, microbial diacylglycerolipids and beta-anomeric self-antigens, which illuminate the TCR docking mechanism. Interestingly, although all glycolipids are presented differently by CD1d the TCR binds with a conserved footprint on top of CD1d, which requires the structural rearrangement of the glycolipid antigen that is sandwiched between both proteins.

In comparison, we have also determined the crystal structure of the type II NKT TCR from the lysosulfatide-specific hybridoma Hy19.3 bound to mCD1d-presented lysosulfatide, which finally allows us to compare the binding mechanism between Type I and Type II NKT cells. The structure suggests that even though Type II NKT cells are diverse in the alpha and beta gene repertoire, they feature several conserved residues that we hypothesize allow the different TCRs to dock in a similar fashion to the CD1d-sulfatifde complex. In summary, the docking mechanism of Type II NKT cells shares both features of Type I NKT cell, as well as conventional peptide-MHC reactive T cells.

The seminar will briefly cover the biology of the NKT cells while focusing on our recent structural findings. It will also address issue related to the formation and isolation of tri-molecular complexes containing lipids, lipid loading into proteins, as well as visualizing lipid-loading efficiencies using native IEF.

Lectures - Sunday, June 24

L5

CRYSTALLOGENESIS METHODS AND STRUCTURAL BIOLOGY – A HISTORICAL OVERVIEW

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Crystallography is one of the oldest sciences and dates back to Antiquity. In biology, protein crystals were obtained for the first time in the 1840s, long before understanding the chemical nature of proteins [1,2]. The birth of structural biology is more recent and happened thanks to methodology-driven efforts combining physics and biochemistry and the insight of a few visionary pioneers dreaming to comprehend the molecules that underlie the basic life processes [3]. Thus, in 1934 Bernal and his student D Crowfoot (Hodgkin) obtained the first X-ray diffraction pattern from protein crystals and noticed that these crystals deteriorate in air. They quickly realized that dehydration was the cause of the damage and therefore that protein crystals should be placed in sealed capillaries for X-ray experiments. In parallel, methods were developed to interpret diffraction data in terms of atomic models [4].

In the 1960's, when the size of the target proteins increased, it was rapidly realized that preparation of suitable crystals represents one of the major bottleneck of structural biology. As a result, the new field of biocrystallogenesis progressively emerged, with both a "practical" and a "basic science" aim. (i) At the practical side, the aim was to provide user-friendly methods for crystal preparation suitable for structure determination. (ii) At the side of basic science, the ambition was to establish rational frameworks enabling to understand and to control the protein crystallization process and also to control and optimize crystal quality.

The historical development of crystallization methods occurred in conjunction with that of genetic methods for protein production and engineering as well as with the easier access to new generation synchrotron X-ray sources for improved data collection. In addition, rational and

high-throughput approaches developed in parallel. The input for structural biology will be discussed and lessons arising from the sociology and the dichotomy between idea- *vs* fashion-driven research will be suggested (an emblematic example comes from protein crystallization in gelled-media, seemingly a novel approach, first described in 1950's by bio-crystallographers [6], rediscovered by physicists of crystal growth in 1988–94's [7,8] and that only presently is slowly infiltrating structural biology laboratories [9]).

Given the increasing number of crystal structures deposited in PDB, it could be argued that protein crystallization no more is a bottleneck in structural biology. Alas this is not the case, since structural biology projects become more challenging (e.g. a need of crystals of molecular assemblages of high complexity) and that unsolved issues still exist (e.g. production of crystals for 4D biology) [3,5].

Selected references:

1. A McPherson (1991) *J Crystal Growth* 110, 1-10;
2. A Ducruix & R Giegé (eds) (1999) Crystallization of nucleic acids & proteins: A practical approach, IRL Press, Oxford, (2nd ed) pp. 1-435;
3. R Giegé & C Sauter (2010) *HFSP Journal* 4, 109-21;
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5. C Sauter, B Lorber, A McPherson & R Giegé (2012) General methods of crystallization; in *'Int. Tables Macromol. Xgraphy'* vol. F chapter 4.1, pp. 99-121;
6. BW Low & FM Richards (1954) *JACS* 76, 2511-8;
7. M-C Robert & F Lefaucheux (1988) *J Crystal Growth* 90, 358-67;
8. J-M Garcia-Ruiz & A Moreno (1994) *Acta Cryst* D50, 484-90;
9. B Lorber, C Sauter, A Théobald-Dietrich, A Moreno, P Schellenberger, M-C Robert, B Capelle, S Sanglier, N Potier & R Giegé (2009) *Prog Biophys Mol Biol* 101, 13-25.

L6

PROPERTIES AND NATURE OF MACROMOLECULAR CRYSTALS

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Macromolecules are large biological molecules that include proteins, nucleic acids and viruses. Macromolecular crystals are comprised of ordered three-dimensional arrays characterized by the disposition and periodicities of its repeating units defined by its symmetry and translation. The fundamental concept of the asymmetric unit, space group, and the unit cell in characterizing a crystal will be discussed. Macromolecules are chiral and as a result, there are only 65 of 230 possible space group symmetries. The general methods to measure the dimensions of the unit cell and how to identify image peaks in reciprocal space will be introduced. In addition, the quality of macromolecular crystals will be discussed in terms of how crystals can be affected by non-identical conformations of macromolecules.

L7

INTERPRETATION OF THE CRYSTALLIZATION DROP RESULTS

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The crystallization drop is full of information for the person who knows what to look for. However, for the inexperienced observer, the interpretation of the phenomena in the crystallization drop is not always a straightforward process. While it is sometimes easy to recognize a crystal, what about all those other solid phases of proteins like oils, precipitates, spherulites, and gels? Are they worth optimizing, or should one continue screening for new conditions? What does it mean when the protein "oils out"? What does phase separation look like and how does it affect the outcome of the experiment? How can you recognize a promising precipitate from a "bad" one? What are the best types of crystals to use as seeds?

This lecture will give present pictorial examples of the most commonly encountered results in crystallization drops and discuss 1) how to recognize the different phenomena, and 2) what to do with them.

The pictorial library of crystallizatiaon drop phenomena can be accessed at: http://xray.bmc.uu.se/terese.

L8

CRYSTALLIZATION AND CRYSTALLOGRAPHIC ANALYSIS IN MICROFLUIDIC CHIPS

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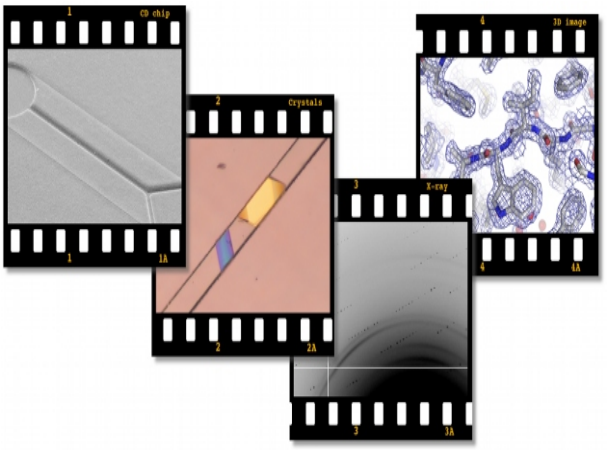
Microfluidic technology has opened new possibilities for the crystallization of biological macromolecules. Indeed, microfluidic systems offer a lot of advantages for crystal growth: they enable an easy handling of nano-volumes of solutions and, thus, extreme miniaturization and parallelization of crystallization assays. In addition they provide a convection-less environment *a priori* favorable to the growth of high quality crystals. Pioneer examples implementing free interface diffusion [1] and nano-batch [2] crystallization in microfluidic chips have already demonstrated the value of this technology, especially for high throughput screening applications in structural genomics.

Examples of microfluidic devices available on the market or in development will be described to illustrate how different steps of a structural study can be carried out 'on chip' from the crystallization to the observation of crystals and their characterization using synchrotron radiation [3,4]. The possibility of replacing conventional crystallization setups with inexpensive microfluidic chips for screening best crystallization agents and for automated crystal diffraction analysis will be discussed.



1. Hansen CL, Skordalakes E, Berger JM, Quake SR. A robust and scalable microfluidic metering method that allows protein crystal growth by free interface diffusion. *PNAS* **99**, 16531-6.
2. Zheng B, Tice JD, Roach LS, Ismagilov RF. A droplet-based, composite PDMS/glass capillary microfluidic system for evaluating protein crystallization conditions by microbatch and vapor-diffusion methods with on-chip X-ray diffraction. *Angew Chem Int Ed Engl.* **43**, 2508-11.
3. Sauter *et al.* (2007). From macrofluidics to microfluidics in the crystallization of biological macromolecules. *Crystal Growth Design* **7**, 2247-50.
4. Dhouib K, Khan Malek C, Pfleging W, Gauthier-Manuel B, Duffait R, Thuillier G, Ferrigno R, Jacquamet L, Ohana J, Ferrer JL, Théobald-Dietrich A, Giegé R, Lorber B, Sauter C. Microfluidic chips for the crystallization of biomolecules by counter-diffusion and on-chip crystal X-ray analysis. *Lab Chip* **9**, 1412-21.

L9

MICROSEED IT! HOW TO GET THE MOST FROM YOUR CRYSTALLIZATION HITS

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Random Microseed Matrix-Screening (rMMS), where seed crystals are added automatically to random crystallization screens, is a significant recent breakthrough in protein crystallization [1]. One industrial group has used the method to solve 38 out of 70 structures generated in four year period, finding particular success with antibody complexes [2]. rMMS not only produces more hits, it also generates better-diffracting crystals - because crystals are more likely to grow in the metastable zone [3].

The theory and practice of the rMMS method will be introduced with case studies, and variations in the method that are helpful for crystallizing membrane proteins and complexes will also be shown [4]. Finally, a simple experimental design for optimizing seeding levels will be presented (this design also has applications for reshuffling the ingredients of several crystallization hits).

1. Allan D'Arcy, Frederic Villarda, May Marsh. 'An automated microseed matrix-screening method for protein crystallization'. Acta Crystallographica section D63 (2007), 550–554. On-line at scripts.iucr.org/cgi-bin/paper?S0907444907007652.

2. Microseed Matrix Screening Crystallization of Antibody Fragments and Antibody-Antigen Complexes. RAMC, Strasbourg, France, 2011. Galina Obmolova, Biologics Research, Centocor R&D hamptonresearch.com/documents/ramc/RAMC2011_T11_Obmolova.pdf

3. Further information on the theory and practice of the MMS method is available at the Douglas Instruments web-site, www.douglas.co.uk/mms.htm.

4. Patrick Shaw Stewart, Stefan Kolek, Richard Briggs, Naomi Chayen, and Peter Baldock. Random Microseeding: A Theoretical and Practical Exploration of Seed Stability and Seeding Techniques for Successful Protein Crystallization. pubs.acs.org/doi/abs/10.1021/cg2001442.

Lectures - Monday, June 25

L10

AN INTRODUCTION TO CRYSTAL MORPHOLOGY AND CRYSTAL GROWTH MECHANISMS

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The description of the shape of the crystals resulting from crystallization experiments is critical information to contrast their reliability and reproducibility. Fortunately, crystallographers have developed since more than one hundred years ago a precise and unambiguous terminology based on crystal symmetry to define crystal shapes in terms of morphology and habits, i.e. in term of combinations of crystal forms and their relative development.

The aim of this lecture is to introduce the main concepts, terms and definitions used in the proper description of morphology of crystals and crystal aggregates obtained in protein crystallization experiments. I will introduce also the different growth mechanisms and growth phenomena that are involved in the morphogenesis of the crystals and how are they connected with the physicochemical crystallization conditions of the solutions from which they growth.

L11

"WHAT TO DO IF EVERYTHING HAS FAILED"

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Protein crystallization projects usually have two stages. The initial one involves screening parameters to find promising lead conditions. Useable crystals may already appear at this stage, but the most typical scenario is that a second round of experiments is required to optimize the potential leads. This lecture will present some of the major considerations in choosing particular strategies or "routes" for screening and optimization. However, since the pathway in a crystallization project often contains dead-ends, the protein crystallizer also needs to be equipped with a plan for dealing with the "detours". When it seems that everything has failed, what are the options left to try?

To address this problem, the following questions will be discussed:

- Can pre-screening the protein buffer improve the protein behavior in the crystallization drops?
- How many conditions should the initial screen contain: 150 or 1500?
- So many crystallization kits! Which one to choose?
- Which is more critical: the choice of precipitant or the kinetic pathway?
- How does one recognize the kind of leads that are worth optimizing? For example, should one try optimizing drops with phase separation or keep screening for new conditions?
- What kind of tools exist for predicting if a protein is going to crystallize? How reliable are they?

This lecture will answer these questions from the perspective of an academic laboratory with little automation and which works on a limited, but very focused, group of targets from *Mycobacterium tuberculosis.*

Bergfors, T. M., ed. Protein Crystallization, 2nd Edition, 2009, International University Press, La Jolla California.

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L12

UNCONVENTIONAL CRYSTALLIZATION TECHNIQUES FOR SCREENING AND OPTIMISATION

Naomi E. Chayen (lecture by Lata Govada)

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The past decade has seen momentous progress in the miniaturisation, automation and analysis of crystallisation experiments. However, high-throughput has not yet resulted in high-output and producing high quality crystals still presents a major barrier to structure determination.

There are no 'magic bullets' that will guarantee the production of good crystals, hence the development of new and improved technologies for obtaining high quality crystals is of crucial importance to progress.

This talk will present strategies for increasing the chances of success and highlight several practical methods that can be used in cases where standard screening and optimization procedures have failed. The methods involve active influence and control of the crystallization environment while the trial takes place, in order to lead crystal growth in the direction that will give the best results. Most of the techniques can be automated and adapted to high throughput mode.

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2. Saridakis, E. and Chayen, N.E. (2009) "Towards a 'Universal' Nucleant for Protein Crystallization *Trends in Biotechnology*27, 99-106.
3. Macromolecular Crystallization and Crystal Perfection". N.E. Chayen, J.R. Helliwell, and E.H. Snell. Oxford University Press, Oxford, UK 2010.

L13

TIPS AND TRICKS FOR PROTEIN CRYSTAL MANIPULATION AND HANDLING

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The possibility to solve any protein structure relies on the ability to obtain a crystal suitable for X-ray diffraction. Obtaining a crystal is just the starting point for a way that sometimes can be very tedious. The next steps will include; i) testing the crystal nature, ii) X-ray diffraction at room temperature, iii) cryo preservation prior iv) low temperature data collection and iv) derivatization. This manipulation can put at risk your crystal quality and therefore the quality of your structure. In this talk we will try to fill the gap between the microscopy and the X-ray "observation" of your crystals with some tips and tricks. We will also see how to include new additives, i.e. cryoprotectant, scatter atoms, etc., into your protein crystal avoiding or minimizing the lost of quality and finally how to perform *in situ* cryo-crystallography from crystals grown by the capillary counterdiffusion method.

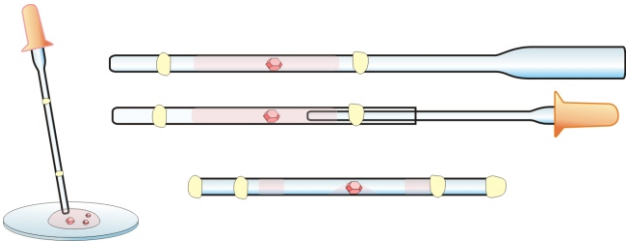

Figure 1. Schematic representation of how to prepare your crystal for room temperature X-ray diffraction test or data collection.

L14

ON THE USE OF ADDITIVES IN PROTEIN CRYSTALLIZATION

Rolf Hilgenfeld

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The addition of certain small molecules to the protein crystallization experiment can have dramatic effects. Such small molecules may be cofactors required by the protein under study, for example nucleotides. These often change the conformation of the protein by binding to specific sites. The same is true for metal ions, for which specific binding is frequently observed in the active sites of proteins, or at their surface. For relatively few of these additives, their effects can be rationally explained today. We have studied the effect of the addition of zinc ions to a number of proteins and found several cases where zinc binding to glutamate or histidine residues at the surface of the protein had the effect of cross-linking the protein molecules in a crystal lattice (see, e.g., Riboldi-Tunnicliffe et al., 2001). A very popular additive is 2-methyl-2,4-pentanediol (MPD) or 1,4-Dithiothreitol (DTT) (Ponnusamy et. al., 2008). MPD is frequently used as the major precipitant in crystallization of proteins and, in particular, nucleic acids. We have studied the structural consequences of MPD binding to proteins in a statistical analysis (Anand et al., 2002). While these studies provided rational explanations for some individual additives, McPherson and Cudney have recently shown that the broad use of mixtures of additives can improve the success rates of protein crystallization trials dramatically (McPherson & Cudney, 2006). These and other ideas will be discussed.

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Ponnusamy R., Moll R., Weimar T., Mesters J.R. & Hilgenfeld R.: *J. Mol. Biol*., in press (2008).

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Lectures - Tuesday, June 26

L15

E.COLI - A FACTORY FOR RECOMBINANT PROTEINS

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The rapid development in recombinant DNA techniques has made it possible to overproduce selected proteins that are normally expressed only at low concentrations, as well as those that have been proven to be difficult to purify to homogeneity from natural sources.

Several alternative systems for the expression of foreign genes have been developed including mammalian cells, insect cells, fungal cells, bacterial cells and transgenic animals or plants. However, most widely used and convenient system for the production of foreign proteins remains that based on the simple Gramm negative prokaryote – *Escherichia coli*. In the last years, many reports showed strong progress in efficient expression and isolation of peptides and proteins using fusion technology with a variety of expression and solubility tags – thioredoxin, MBP, GST, NusA, ubiquitin, SUMO, GB-1, etc. Generally there is no ideal host and no expression system that meets all needs. However, we successfully used the redesigned Ub (ubiquitin) possessing an N-terminal His tag on the most proteins that failed to be expressed before.

The purpose of this talk, therefore, will be to provide background information and at the same time address on practical solutions to the most common problems in expression of heterologous proteins. This should enable you to design a competent strategy in your own future research. Some of the major factors will be reviewed in this lecture, which has to be considered in selecting an *E. coli* expression strategy (for example: cloning strategy, functional activity of the proteins, medium engineering, removing fusion tags troubles). Furthermore, tips and tricks will be discussed how to provide enough functional material for biological studies.

L16

THE ROAD FROM PROTEIN EXPRESSION AND PURIFICATION TO PROTEIN CRYSTALLIZATION

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Many efforts have been performed to understand the relationship between the structure of proteins and their biological function. In addition, the huge number of candidate proteins generated by genomics programs has increased the interest in all aspects of protein expression and purification. Proper expression and purification techniques are essential for the large-scale production of pure proteins needed for 3-D structure determination. One can obtain high levels of expression by choosing good host strains, vectors, and growth conditions. However the purification flowchart that leads you into a successful crystallization experiment will have to be established by yourself for every single new protein you might get. Commonly questions one should answer before starting a new protein crystallization project include: should my protein(s) be expressed in bacteria, in yeast, in insect cells or in human cells? Which expression vector should I use? If I choose bacterial expression, which strain(s) should I use? Should I express the full-length protein or a fragment? Should my protein be tagged, and which affinity tag is the best? Do I need to remove the tag? Which buffer should I use? What is a good purification strategy for my protein?

Unfortunately, because every protein is different, you will not be able to answer these questions *a priori*, and you will have to work out the purification protocols and strategies for each individual protein. The better the protocol is established the higher is the chance you will get good crystallization hits. In this talk, you will get an illustration of some of the most commonly used strategies helping the researchers in such a complicated task.

L17

PREPARATION OF PROTEIN SAMPLES FOR CRYSTALLIZATION EXPERIMENTS

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Protein crystallization process is influenced by a large number of various factors and one of the most important is the property of the protein sample to be crystallized. Preparation and characterization of the protein sample plays a crucial role in protein crystallization.

In the lecture, the most widely used techniques to judge evaluate protein sample purity and quality before crystallization experiments will be reviewed and discussed. For crystallization trials highly pure and homogeneous protein sample is usually recommended, however, if larger amount of protein is available 'impure' protein sample can be also screened. Guidelines and tips for protein handling before crystallization trials will be addressed.

Common methods to analyze protein purity and stability will be described: (a) SDS polyacrylamide gel electrophoresis (PAGE), (b) native PAGE, (c) isoelectric focusing, (d) size exclusion chromatography (gel filtration), (e) mass spectrometry, (f) dynamic light scattering (DLS), and (g) differential scanning fluorimetry (DSF).

Recommended protein concentration for initial crystallization screening is in range of 5 - 20 mg. The higher protein concentration provides more opportunity for crystal nucleation to occur but, on the other hand, also can cause protein aggregation. The best concentration is usually tuned as one of the variables during optimization procedure. All components of the protein buffer should be carefully considered since they might influence crystallization. Storage conditions have to be checked experimentally for each protein, but most protein can be stored at -70 C or 4°C. Lyofilization should be avoided and if inevitable, extensive dialysis before crystallization is recommended.

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L18

PROTEIN AS THE MAIN VARIABLE IN CRYSTALLIZATION

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Preparation of high quality protein crystals is essential for the structure determination using X-ray techniques. Statistics from the projects of structural genomics shows that the success rate of high-throughput crystallization is only 10-30 % and thus preparation of protein crystals becomes the rate-limiting step.

Crystallization is influenced by many parameters, from which the most important one is the protein itself, its purity, homogeneity and specific properties, namely its propensity to form crystals. Protein crystallizability may be enhanced by the methods of molecular biology. This may involve the preparation of proteins with various kinds of fusion partners or tags, removal of their most flexible parts (N- and C-termini or flexible loops), increasing the homogeneity by modifications of free cysteines or potential sites of glycosylation, replacement of unfavourable amino-acid residues at the surface of the molecule, etc.

Requirement of protein purity and homogeneity will be discussed and stressed. The influence of protein modifications on its crystallizability and/or crystal packing and quality will be documented on results obtained in our laboratory and examples from literature. Some rational approaches and strategies oriented on enhancing the protein crystallizability as well as the possibility of its computational prediction will be presented.

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Lectures - Wednesday, June 27

L19

COUNTERDIFFUSION METHODS FOR PROTEIN CRYSTALLIZATION AND SCREENING: GELS, CAPILLARY VOLUMES AND MICROGRAVITY

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Protein crystals are grown always from aqueous solutions and therefore actual crystallization experiments are affected by gravity. Typically, protein crystallization experiments display phenomena such as evaporation, sedimentation and convective mixing, that alter the homogeneity of the volume of the solutions and that provoke sometimes unwanted complex dynamics, which are difficult to control. Therefore, removing gravity driven phenomena, such as sedimentation and convection, is a way to control the space and time evolution of the experiments but it also open an alternative way to design protein crystallization experiments, by coupling diffusion mass transport with chemical precipitation.

In this talk I will introduce the fundaments of the crystallization method named counterdiffusion, which is based on the coupling of the precipitation and the diffusion transport of the molecules of proteins and antisolvents used to reduce the solubility of the protein. The technique can be performed with different implementations, namely in gels, in capillary volumes and in low gravity environments in space. I will introduce first the fundamental basis to understand the time evolution of the supersaturation and supersaturation rate along the crystallization reactor and how this can be used to design very effective screening of the crystallization conditions. Then, I will explain why and how this technique can be used for the optimization of crystal size and crystal quality of proteins. Finally, the different implementation will be shown with the help of videodemonstrations.

F. Otálora, J. A. Gavira, J. D. Ng. and J. M. García-Ruiz. Counterdiffusion methods applied to protein crystallization. *Progress in Biophysics and Molecular Biology* **101** (2009) 26-37.

J. M. Garcia-Ruiz and L. A. González-Ramírez, Capillary counterdiffusion experiments with prefilled Granada Crystallization Boxes. *Protein Crystallization*: Second Edition Terese Bergfors, Ed; IUL Biotechnology Series. International University line 2009; pp 395-400.

L20

FLUORESCENT METHODS FOR PROTEIN CRYSTALLIZATION SCREENING

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Fluorescence can be a powerful tool to aid in the crystallization of proteins. We are currently using two approaches, fluorescence anisotropy and trace fluorescent labeling, each with their associated advantages. Both methods rely on the covalent labeling of the protein to be screened with removal of non-bound free probe. For the trace labeling approach the protein is covalently derivatized with a high quantum yield visible wavelength fluorescent probe. The final probe concentrations are typically < 0.25% of the protein molecules labeled, which has been shown to not affect the crystal nucleation or diffraction quality. The labeled protein is then used in a plate screening experiment in the usual manner. Protein crystals show under fluorescent illumination as the brightest objects in the well. This approach also finds 'hidden' leads, in the form of bright spots, with ~30% of those lead found being optimized to crystals in a single pass optimization trial. Visible fluorescence also enables selection of conditions that bypass interfering substances and greatly reduces materials considerations. The fluorescence anisotropy approach is based upon labeling of the protein with a long-lifetime probe, then preparing a series of dilutions of the protein in each precipitant solution of a screen. The fluorescence anisotropy of the solutions is determined for each condition and the trend evaluated. The anisotropy, a measure of the rotational rate of the probe in the solution, is a function of the mass of the labeled material, and thus the data reflects the proteins self association with respect to the solution conditions. A major advantage of this approach is that it can 'find' lead conditions from solutions that are clear in a plate assay. The disadvantage of the anisotropy approach is that it requires more complex solutions preparation, requiring accurate solution dispensing in the 10's of nL volume range, and more complex data analysis.

L21

DLS MEASUREMENTS PRIOR TO CRYSTALLIZATION EXPERIMENTS

Karsten Dierks[1], Arne Meyer[1], Howard Einspahr[2] and Christian Betzel[3]

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Automated methods to crystallize macromolecules are widely used and can easily generate thousands of crystallization droplets. Nevertheless, evaluation of crystallization experiments to find optimal growth conditions remains a bottleneck. We have investigated and improved methods allowing to analyze the process of evaluating crystallization experiments and identifying crystal growth conditions. Besides imaging drops, two methods have emerged as most promising. One is dynamic light scattering (DLS), which has already many applications, but which we have found useful for detection of aggregation and nucleation in droplets as well as in counter diffusion capillaries [1-3]. The other is the use of a combined white/UV illumination for microscopic determination of whether crystal-like objects are biomolecular and identification of crystals in crystallisation set ups [4]. DLS is a widely accepted method to determine the size and mode of aggregation of proteins and other biomolecules in solution, but its use has so far been limited because the need to make measurements in cuvettes required rather large sample volumes. DLS is also a well established method to optimise protein solution quality prior to crystallisation experiments by analysing the dispersity. Protein crystallisation experiments are usually carried out in multi-well plates with droplet volumes in the range of 0.5 to 10 µl. We will describe a relative new method to image, measure and analyse the protein particle size directly in drops and in particular to investigate the stage of nucleation and the progress of crystal growth by *in-situ* DLS, i.e. directly in the droplets. This has several advantages: no additional pipetting is necessary to perform measurements; the crystallisations process can be monitored online *in situ*, without interruption; measurements can be taken from even small volumes. This new DLS technique has been adapted to two automated CCD-camera-based plate-screening systems (Spectro-Imager 501, and Spectrolight 600, Molecular Dimensions, UK) allowing monitoring and evaluation of the entire process of crystallisation in an automated way. The data obtained provide information to understand in detail the process of crystal growth. We will also describe a method to support the identification of protein crystals, exploiting the fact that most proteins and other biomolecules fluorescence when illuminated with UV light. The imaging and scoring systems mentioned before incorporate all of the techniques described above in one device. Images taken from various

droplets/set ups will be presented along with corresponding DLS measurements. Details will be presented.

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L22

PUBLICATION OF SCIENTIFIC RESULTS WITH EMPHASIS ON CRYSTALLIZATION COMMUNICATIONS

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The crystallization communication is often the beginning crystallographer's first encounter with scientific publication. The fundamental principles of scientific publication will be presented with special emphasis on crystallization results either as a crystallization communication or as part of a structure report. Included will be an introduction to *publBio*, a collection of novel web-based tools for authors developed by IUCr Journals to facilitate drafting of crystallographic publications and speed editorial processing after submission.

L23

LARGE VOLUME CRYSTAL GROWTH IN RESTRICTED GEOMETRY FOR NEUTRON CRYSTALLOGRAPHY

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Neutron Macromolecular Crystallography (NMC) is the prevalent method that accurately determines the positions of hydrogen atoms in macromolecules. There are two major limiting factors in determining protein structure with neutron diffraction; one is obtaining large crystal volumes and the other is the extended experimental duration. This lecture will demonstrate that crystallizing hyperthermophilic proteins by counter-diffusion crystallization can produce suitable protein crystals for NMC. Counter-diffusion crystallization in restricted geometry, such as in capillary tubes, allows diffusion to govern mass transport processes so that a spatial-temporal gradient of supersaturation is formed along its length. Consequently, proteins can crystallize in an optimized supersaturated condition where a single crystal can fill up the diameter of the capillary. We have employed the same process in capillary vessels having diameters exceeding 1mm while minimizing the ratio between buoyant and viscous forces. As a result, protein crystals suitable for neutron crystallography can be grown in this configuration with volumes greater than 1mm^3 while eliminating invasive crystal manipulation. Using crystals grown by this method, we have undergone neutron diffraction analysis of recombinant proteins from a hyperthermophilic archaeon. These proteins are excellent crystallization targets because of their thermal and mechanical stability and high propensity to crystallize. As an initial proof of principle, a novel inorganic pyrophosphatase (IPPase) was among the first of these proteins to be grown in large diameter capillaries resulting in a 9mm^3 volume crystal. The next generation neutron beamlines at the Spallation Neutron Source (SNS) along with an effective counter-diffusion crystallization procedure can improve structures of a diverse range of proteins of interest to the crystallographic community. Thus the crystallization of macromolecules can grow to volumes never before achieved, potentially leading to enormous increases in the number of neutron crystallographic protein structures.

Lectures - Thursday, June 28

L24

NUCLEATION OF PROTEIN CRYSTALS: MECHANISMS AND SUGGESTED CONTROL STRATEGIES

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There are several fundamental aspects with crucial practical implications, related to the nucleation of protein crystals. The first one is, assuming that a crystal form with a sufficiently low free energy exists, how does one make sure that nuclei of this form appear within reasonable time in a tested solution? Another one is how does one ensure that just a few, and not numerous interfering crystals reproducibly form in a crystallization experiment? Furthermore, if several crystal polymorphs with different structures are possible, a crystallographer may want to select the one providing better detail of the protein atomic structure.

Experiments, simulations, and theoretical work, carried out in our and other laboratories in recent years, have revealed intimate details about the nucleation mechanisms of protein crystals. It was shown that crystals of many proteins follow a two-step mechanism of nucleation, whereby the formation of an ordered nucleus is preceded by a metastable dense liquid droplet. It was found that such droplets exist in proteins solutions, even if they are undersaturated with respect to any solid phase. It was shown that the formation of the metastable dense liquid droplets is an intrinsic property of the solution, which is a consequence of the structuring of the water molecules at the protein molecular surface. A kinetic theory, linking the properties and the volume occupied by the dense liquid droplets to the crystal nucleation rate has been developed.

The most significant practical consequence related to these theoretical insights is that by controlling the formation and the properties of the dense liquid droplets, several aspects of the nucleation protein crystals: number of crystals, reproducibility of nucleation, polymorph selection, can be controlled. These and other issues of interest to the practicing crystallographers will be discussed.

Vekilov, P. G. Nucleation. *Crystal Growth & Design* **10**, 5007-5019, (2010).

L25

PREPARATION OF MICRO- AND NANO-CRYSTALS FOR FUTURE FREE-ELECTRON-LASER AND SYNCHROTRON RADIATION SOURCES

Rudolf Koopmann[1], Michael Duszenko[1], Herny Chapmann[2,3], Lars Redecke[4], Arne Meyer[5], Karsten Dierks[5], Howard Einspahr[6], Rolf Hilgenfeld[7] and Christian Betzel[8]

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Growth and preparation of high quality micro-crystals optimal for data collection experiments at modern micro-beam insertion-device synchrotron (SR) beamlines and growth of nano-crystals required for data collection at future Free-Electron-Laser (FEL) beamlines is a new challenging task. In the field of protein crystallization several fully automated instruments are available today and the search for crystallization conditions of macromolecules can easily carried out. Nevertheless, to identify optimal growth conditions to obtain high quality X-ray suitable crystals still remains a bottleneck in most cases [1,2]. Considering the tremendous advantages of the new and upcoming high brilliant SR- and FEL- radiation sources, allowing to collect diffraction data from micro- or nano- crystals by conventional single crystal diffraction or via the new method of Serial Femtosecond Crystallography (SFX) [3]

new crystallization and crystal scoring techniques need to be established.

SFX offers new possibilities to analyse proteins that do not form crystals suitable for conventional X-ray diffraction in vitro and will open new routes in structural biology [4].

To meet future crystal requirements at FELs we followed two approaches to produce high quality nano- and micro- crystals during the last years. In one approach we optimized the growth of nano sized crystals *in vivo,* in cells [5] and in a second approach we developed a advanced hardware combination allowing the controlled optimization of a single drop vapour diffusion experiment for production of nano- and micro- crystals [6]. Details and examples will be presented.

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L26

HOW TO SEE HYDROGENS TOO – INTRODUCTION TO NEUTRON CRYSTALLOGRAPHY

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The most common method to study enzymatic mechanisms from a structural point of view is X-ray crystallography. As it is based on the scattering of X-rays from electrons, hydrogen atoms with only one electron are particularly difficult to observe. Even in the highest resolution X-ray structures of proteins determined thus far, not all the hydrogens are visible in the electron density maps. Whether a hydrogen atom is observed or not is highly dependent on the atomic displacement factor (or B-factor) of the atom it is bound to, which in practice means that many of the biologically more interesting polar hydrogens in the side chains are not observed, even at subatomic resolution. The same limitation applies to non-covalently bound ligands in which the polar hydrogen atoms are rarely as ordered as, for example, main chain amide hydrogens.

One method to observe the protons more directly is nuclear magnetic resonance spectroscopy (NMR). The ^{13}C resonances of carboxylates or ^{15}N resonances of histidine change as a function of protonation, so an NMR titration in which these resonances are observed can determine the pK_as of individual residues and hence their protonation states at the pH of interest. The major limitation of NMR is that the molecule has to tumble sufficiently rapidly in solution, which imposes a practical size limitation. As the size of the molecule increases, the spectra grow more complicated and elaborate labelling schemes are required to resolve and interpret them.

The most unambiguous method for determining the proton positions in the protein is neutron crystallography [1-2]. As neutrons are scattered from the atomic nuclei rather than the electrons, the scattering lengths of hydrogen and the other elements commonly found in proteins (*i.e.* carbon, nitrogen, oxygen, sulphur) are of roughly equal magnitude. This 'high visibility' of hydrogen with neutrons allows the positions of the protons also in water molecules to be determined at much lower resolutions (< 2.5 Å) than are necessary with X-rays. Despite this clear benefit of neutron crystallography, currently only around 30 neutron macromolecular structures have been deposited in the Protein Data Bank (http://www.rcsb.org/pdb). This is due to various difficulties associated with neutron crystallography, such as the growth of large and well-ordered crystals under deuterated conditions [3-4], that make a successful experiment more demanding than its X-ray counterpart. Recent key developments in instrumentation, detection systems and sample preparation have dramatically reduced the sample volume and data collection times required and this has led to an expanding field addressing larger and more complex problems and employing more sophisticated refinement methods [5-6].

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L27

SCREENING THE DIFFRACTION QUALITY OF PROTEIN CRYSTALS

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What is a good crystal? Once you have obtained a crystal of suitable size, with nice edges and good morphology, there is more to be done in order to prepare for the collection of a good quality dataset.

This talk will provide an introduction to the X-ray screening of protein crystals in order to select those with the best chance of producing good quality datasets. The discussion will include the visual analysis of images to estimate diffraction quality and identify features in the diffraction pattern, crystal indexing, and the treatment of twinned crystals. An overview of the procedure for finding the best cryo-protection conditions is included. Finally we will provide an overview of the considerations involved in setting up a good data collection experiment, based on crystal information obtained, the instrument and the kind of experiment that is to be performed.

L28

CROSS-INFLUENCE PROCEDURE AS ALTERNATIVE CRYSTALLIZATION TECHNIQUE

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In general, the crystallization of proteins is a very complex process. Experiences of many scientists point out that majority of proteins is difficult to crystallize and even if a protein tends to crystallize relatively easily there are many parameters that must be taken into account. There are multiple reasons that point out the difficulty of protein crystal growth. Therefore, finding of successful crystallization conditions for a particular protein remains a highly empirical process. During optimization a variable set of parameters is screened to determine appropriate conditions for nucleation and growth of single crystals suitable for X-ray diffraction analysis. In parallel to modern high-throughput approaches used in the protein crystallization, in recent years we performed basic research on physico-chemical properties and molecular interactions influencing crystal growth. Empirically, we have explored another tool useful for optimization strategy that was first described by Tomčová and Kutá Smatanová (2007). A new crystallization procedure modifying protein crystal morphology, internal packing and influencing crystal growth was tested particularly. For the first time the metal ion salts were added simultaneously to the protein drop and even to neighboring drops to allow a cross-influence effect of additives during crystallization experiment. The presence of metal ions significantly influences the crystal growth, as the modification of crystal morphology and internal packing were observed. This newly discovered cross-crystallization method (Tomčová & Kutá Smatanová, 2007; Tomčová et al., 2006) was called Cross-Influence Procedure (CIP).

Ivana Nemcovicova and Ivana Kuta Smatanova: Chapter 11: Alternative Crystallization Technique: Cross Influence Procedure (CIP). In the *Crystallization and Materials Science of Modern Artificial and Natural Crystals*, Pages 249-276, Edited by: Elena Borisenko, ISBN 978-953-307-608-9, Publisher: InTech (2012).

Ivana Tomčová and Ivana Kutá Smatanová: Copper co-crystallization and divalent metal salts cross-influence effect – a new optimisation tool improving crystal morphology and diffraction quality. *Journal of Crystal Growth* **306**, 383-389 (2007).

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