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New directions in conventional methods of protein crystallization

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ABSTRACT

Novel strategies and techniques that are based on conventional crystallization methods for crystallizing proteins are described and discussed. New directions for rendering proteins and protein complexes to become more amenable to crystallization are also presented.

1. Introduction

The advent of high-throughput methods in structural genomics has streamlined the process from protein expression, purification and crystallization to target selection and data collection. Current high-throughput robotics permit the miniaturization of experiments and allow the set up of up to 10^5 crystallization trials per day (Stevens, 2000; Mueller et al., 2001; Manjasetty et al., 2008). However, compared to the large number of high-resolution small-molecule crystallographic data deposited in the Cambridge Crystallographic Data Centre (CCDC) i.e. 469,611 (latest update was on January 2009), relatively few protein structures (61,418 up to November 16, 2009) are available in the PDB (Blundell et al., 2002a; Fogg and Wilkinson, 2008). This is a reflection of the difficulty of obtaining good quality diffracting crystals of proteins and other macromolecules. This situation has motivated researchers to come up with novel approaches as well as a wide range of modifications of established crystallization methods in order to increase the chances of forming single crystals suitable for structural studies.

All methods of protein crystallization involve a phase transition in which protein molecules eventually come out of the solution to form crystals when the solution is brought into supersaturation (Arakawa and Timasheff, 1985a; Boistelle and Astier, 1988). Following nuclei formation, the concentration of protein in the solute gradually decreases, driving the system into the metastable zone where growth occurs without the formation of further nuclei (Feher and Kam, 1985; Feher, 1986; Ducruix and Giege, 1992; Garcia-Ruiz, 2003). From a practical perspective, this physico-chemical behaviour opens the possibility of manipulating the system at the early stage of nuclei formation and the initial steps of crystal growth (Bergfors, 2007). However, excessive nucleation may occur if supersaturation is very high, which leads to the formation of hundreds of small crystals resulting in a lack of space for the crystals to grow undisturbed (Nanev, 2007a) and of the crystals competing for protein from the solution. This results in the accumulation of structural defects, leading to low order in the crystal as well as to premature cessation of crystal growth. High supersaturation also implies the rapid incorporation of impurities (this includes partially folded molecules, foreign molecules, proteolysis products, etc.), which would otherwise have been excluded from the growing crystal (Chernov 2003).

There are two types of nucleation: homogeneous and heterogeneous (McPherson and Shlichta, 1988; Nanev, 2007b; Saridakis and Chayen, 2009). Homogeneous nucleation arises from a random event when a sufficient number of molecules cluster together at the same time and in the same region of the solution to form a critical nucleus. Heterogeneous nucleation can be defined as the formation of critical nuclei on particles or surfaces that facilitate the process, usually by attracting the molecules electrostatically, hydrophobically or through specific interactions that can take place at metastable conditions. When nucleation is homogeneous, the extent of nucleation is proportional to the volume of the droplet while in heterogeneous nucleation it is proportional to the area of the solution/nucleant interface.

According to the two-step model of protein crystal growth, the formation of a cluster of solute molecules of a critical size is followed by the reorganization of the cluster into an ordered structure (Feigelson, 1988; Vekilov, 2005). Recent experimental and theoretical

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studies have confirmed the applicability of the two-step mechanism to both macromolecules and small organic molecules, suggesting that this mechanism may underlie most crystallization processes from solutions (reviewed by Erdemir et al., 2009).

Since protein crystallization remains a major hurdle in Structural Biology, the present work reviews the state of art of conventional methods of crystallization -such as vapor diffusion and batch- and discusses some of the novel modifications to these methods that improve the chances of forming good quality single protein crystals for structural studies.

2. Conventional crystallization methods

2.1. Vapor diffusion

Methods based on vapor diffusion have produced more crystallized macromolecules than all other methods combined and are firmly established as the most widely used in protein crystallization. A smaller number of proteins have been crystallized with the batch and dialysis methods (McPherson, 1995; Chayen and Saridakis, 2008) and even less have been crystallized with methods based on free-interface diffusion (Koszelak et al., 1995; Chayen and Saridakis, 2008).

The conventional set up of the vapor-diffusion method consists of an aqueous drop where the protein and the crystallization agents are mixed in an amount lower than that required for the formation of crystals. The crystallization mixture is placed in the vicinity of a reservoir that contains a high concentration of salt or other non-volatile precipitating agent where it is equilibrated against the reservoir. Slow diffusion of water from the crystallization solution into the concentrated solution of salt is due to the difference in osmotic pressures of the two solutions. This diffusion leads to a decrease in volume of the crystallization mixture (hence, to the gradual concentration of the crystallization solution) and therefore to a sufficient increase in the supersaturation of the crystallization solution for the nucleation of the protein crystal to occur. Crystallization conditions are usually identified by performing a large number of trials in which variable ratios of solutions of a protein, precipitating agents, and additives are pipetted together by hand typically 1–4 μL droplets or with a robotic dispenser (10 to ≈300 nL droplets) (Zheng et al., 2005). An individual crystallization trial proceeds through a range of conditions, thereby conducting a self-screening process.

The vapor diffusion method permits acceleration of the nucleation of protein crystals by varying the distance between the reservoir and the crystallization drop (Cudney et al., 1994; Luft et al., 1994). It also allows modification of the composition and/or the concentration of the components in the trial without disturbing the drop. However, because vapor diffusion is a dynamic system where conditions are continuously changing during the crystallization process, it is often difficult to determine the particular stage of the experiment that can be optimized. The addition of an oil barrier over a reservoir of a vapor diffusion trial is useful to approach supersaturation more slowly (Chayen, 1997).

Sitting- and hanging-drop vapor diffusion methods are easy to perform and allow flexible screening with minimal sample volume. The sitting-drop technique has benefits over hanging-drop plating, such as cost and time efficiencies, but crystals often adhere to the hardware surface. The hanging-drop technique reduces the occurrence of hardware crystal adherence and improves the crystal shape and size because of the inverted position of the drop, but this method has the disadvantage of requiring silicone grease and a siliconized cover slip. A simple adaptation of a conventional sitting-drop plate to a hanging-drop set up has been introduced by Whon et al. (2009) by incubating the sitting-drop plates upside down. This is achieved by using agarose gel to solidify the reservoir solution of the sitting-drop plates.

The hanging-drop method permits the transference of a cover slip containing the crystallization drop from one reservoir to another without disturbing the drop. This provides more flexibility for changing the conditions than the batch method (see below) where any change other than temperature involves disturbance of the crystallization drop itself. Higher quality crystals have been obtained by transferring cover slips from nucleation to growth conditions (e.g. Chayen, 2005).

2.2. New seeding procedures

A popular strategy for the optimization of crystallization conditions in vapor diffusion is seeding. There are many different protocols and strategies for doing this. We recommend the excellent review by Bergfors (2003) for a detailed account of seeding methods for protein crystallization. Among the new trends in protein crystallization by vapor diffusion, the seeding method referred to as 'microseed matrix screening' is particularly attractive (Fig. 1). The method permits the use of poorly diffracting crystals to seed into similar but non-identical conditions. Interestingly, such strategy resulted in the formation of better quality crystals with a 10% reduction of the unit cell (Ireton and Stoddard, 2004). More recently, D'Arcy et al. (2007) have developed a simple, automated microseeding technique that is based on the microseed matrix screening of Ireton and Stoddard. The method consists of the addition of seeds into the screening procedure using a standard crystallization robot and has a genuine potential to improve hit rates in early stages of screening. At the same time, the method represents an apparent paradigm for the understanding of the mechanisms of crystal growth. This is because conventional theory of crystal growth dictates that seeds should be introduced into a pre-equilibrated mixture of mother liquor and protein to ensure the microcrystalline seeds remain out of solution, otherwise they are expected to re-equilibrate and dissolve. Although it is possible that in some conditions seeds are preserved as a result of the high concentration of the precipitant agent present in the reservoir, this is unlikely the case where large dilutions of the seed are performed.

This paradigm has been investigated further by St. John et al. (2008). The authors found that the inherent chemical shift in all conditions in a sparse-matrix screen is due to the uniform addition of mother liquor to stabilize the microcrystalline seed stock. This implies that in some cases crystal growth can be induced by the chemical shift caused by addition of the mother liquor rather than the "seed" itself, thus the formation of protein crystals may occur because the stabilizing solution is always very similar to the condition that produced the initial crystals (Fig. 1). The chemical shift resulting from the addition of mother liquor may also play a role in the successful crystallization of proteins seeded with 'oily drops' that are rich in protein (Kuznetsov et al., 2001). One example is the crystallization of the cytochrome domain of cellobiose dehydrogenase (Hallberg et al., 2000; Bergfors, 2003). Interestingly, St. John et al. (2008) also noted that sometimes seeded drops produced more yet smaller crystals, suggesting that seeds were stable under such conditions, whereas in some other crystallization conditions addition of mother liquor did not yield crystals at all. Taken together, these findings show that in some conditions the chemical shift caused by addition of the mother liquor might be sufficient to induce crystal growth while in other conditions seeds may be preserved and essential for a crystal to growth. From a practical perspective this is good news because induction of crystal growth as the result of a chemical shift expands significantly the number of potential hit conditions.

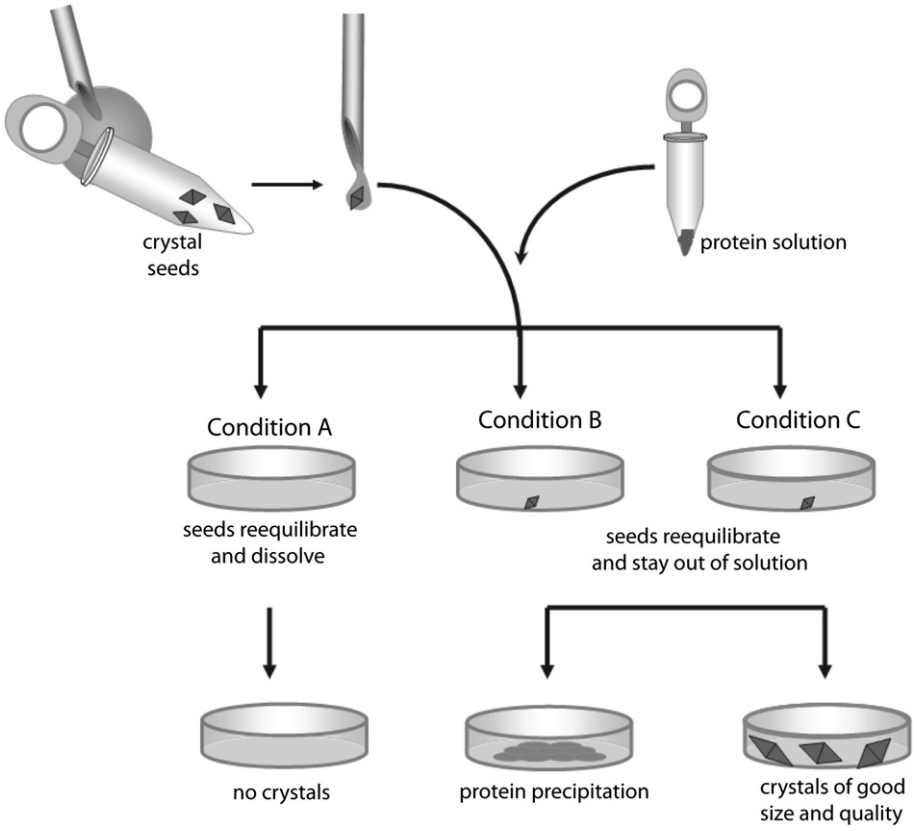

Fig. 1. Microseed matrix screening. Poorly diffracting crystals and/or microcrystals can be used to seed similar or completely different crystallization conditions. Seeding is usually followed by the addition of the protein solution to the crystallization mixture.

2.3. Batch crystallization

Although vapor diffusion is the most commonly used technique for crystallization, it has several problems such as changes in drop volume, changes in pH due to volatile ions, slight temperature change that can cause dissolution of crystals and the composition changes during crystallization process. Many of the difficulties associated with vapor diffusion can be overcome by using the batch method, in which the protein to be crystallized and the crystallizing agents are mixed at their final concentrations at the start of the experiment.

The original batch method requires millilitre quantities of material, thus a micro-scale batch experiment called microbatch was developed (Chayen et al., 1990) whereby nanolitre volume crystallization drops are dispensed and incubated under low density (0.87 g cm^{-3}) paraffin oil in order to prevent their evaporation and also protects them from evaporation, contamination and physical shock (Chayen, 1997). Once initial hit conditions are identified, the quality of crystals is optimized by exploring a subset of conditions throughout the area of interest.

Microbatch is the simplest crystallization method and therefore lends itself easily to performing high-throughput trials (Chayen, 2007). Current robots can dispense microbatch trials down to 1 nL volumes.

The fundamental difference between the vapor diffusion (and other diffusion techniques such as free-interface diffusion) and microbatch is that diffusion methods are dynamic systems in which conditions are changing throughout the crystallization process, while in batch, the samples are mixed at their final concentration at the start of the experiment thus conditions are constant within a normal time (1–3 weeks) of a crystallization experiment. Due to the stability of conditions in microbatch, there are usually no changes in drop volume, pH nor dissolution of crystals (Chayen, 1998).

A problem which arises when screening in microbatch is that because supersaturation is achieved upon mixing there is less exploration of the phase diagram compared to vapor-diffusion trials. Consequently several batch trials may be required to replace a single vapor-diffusion experiment. The application of 1:1 (v/v) mixture of silicone oil and paraffin oil ("Al's Oil") to cover the drop (D'Arcy et al., 1996) overcomes this problem as the mixture of oils allow the concentration of the crystallization drop thereby facilitating a screening effect similar to vapor diffusion. This method has been shown to find more hits than using pure paraffin and is widely used in conventional microbatch experiments (Baldock et al., 1996). Even more hits can be found by using pure silicone oil to cover the drops in place of the silicone/paraffin mixture (D'Arcy et al., 2003a) and the hits also appear much more rapidly. The disadvantage of using pure silicone is that crystals do not last for long due to the subsequent drying out of the crystallization drops. Silicone oil for screening is particularly suitable for use with automatic imaging systems that have become an integral part of high-throughput platforms.

It has been concluded that for screening purposes it is preferable to use silicone oil or a mixture of paraffin and silicone oils (D'Arcy et al., 1996, D'Arcy et al., 2003a) while for optimization, where the conditions need to be known and stable, the trials must be covered by paraffin or poly-fluorinated oils (Bolanos-Garcia, 2003, 2005).

The microbatch method can also be used to crystallize membrane proteins (Chayen, 1997). Detergents like fos-choline-12, n-octyl-beta-D-glucopyranoside and dodecyl maltoside, which are commonly used to solubilize integral membrane proteins, are

compatible with the microbatch method as the oils show very low partitioning coefficients (Loll et al., 2003; Barends and Dijkstra, 2003).

A different combination of microbatch and oils consists of the use of gelled microbatch drops to grow crystals under an oil layer. In addition to the advantage of consuming very small quantities of sample compared to single gel-based techniques, the combined method enables the dispensing of numerous trials automatically (Chayen and Saridakis, 2002) and is amenable to high-throughput experimentation. Another modification to the conventional microbatch method allows the regulation of the rate of water evaporation and thus the fine tuning of the crystallization conditions and the prevention of desiccation of the drops (Brumshtein et al., 2008).

Most proteins that can be crystallized by vapor diffusion can also crystallize with the microbatch method, often after slight modification of the original crystallization conditions. Exceptions are crystallization conditions in which oil-soluble precipitants are used (Chayen, 1997). Empirical rules for converting crystallization conditions from one method to another include lowering the concentration of precipitant in microbatch to approximately 10–20% less than that used in vapor-diffusion trials. For proteins where crystallization takes place very rapidly, as in the case of thaumatin, lysozyme and ferritin which crystallize within a few hours, significantly lower concentrations of protein and/or precipitant should be used in batch. The optimal concentrations of buffers and additives usually remain the same in both methods (Chayen, 1998).

2.4. Crystallization of membrane proteins

From the crystallization point of view, there is no significant difference in the methods for crystallizing membrane proteins. The difficulties in crystallizing these proteins are mainly due to the inherent qualities of the membrane proteins. Special screens based on previous successes with membrane proteins have been developed (e.g. Iwata, 2003) and commercialized, such as the MembFac (Hampton Research, USA; http://hamptonresearch.com) Memstart and Memsys (Molecular Dimensions, UK; http://www.moleculardimensions.com/uk), but often these proteins crystallize in the standard screens used for soluble proteins. As for soluble proteins, the most commonly used approach for crystallizing membrane proteins relies on conventional vapor diffusion methods. However, microbatch, microfluidics and lipidic cubic phase or lipidic mesophase are also used successfully (Chayen, 2009).

In addition to the continued use of conventional methods, new directions in the past few years have involved miniaturised methods that exploit the ability of lipids to form liquid crystals or mesophases and to reconstitute membrane proteins (Cherezov et al., 2003; Nollert, 2005; Caffrey, 2009). A robotic system for crystallizing membrane proteins in lipidic mesophases was reported by Cherezov et al. (2004). The most impressive breakthrough using the lipidic cubic phase for crystallization has very recently led to the structure determinations of G protein-coupled receptors (Weber et al., 2007; Jaakola et al., 2008; reviewed by Hanson and Stevens, 2009).

3. Biophysical characterization prior to crystallization

Many of the central goals of structural, functional and chemical genomics such as fragment-based drug discovery and protein-based screenings for small molecule inhibitors depend on the availability of purified and active proteins. Because the biophysical characterization of protein solutions is mandatory before the setting up of crystallization trials, a vast range of biophysical techniques and methods are customarily employed to assess the quality and stability of protein solutions. Two techniques of wide use are emission fluorescence and light scattering (Malkin and McPherson, 1994; Senisterra and Finerty, 2009). The former technique allows measurement of changes in fluorescence as a protein unfolds and/or undergoes important conformational changes induced by ligand binding. Dynamic light scattering (DLS) is a useful tool for non-invasive *in situ* monitoring of crystallization trials because it detects the formation of aggregates or nuclei before they become visible with a light microscope (Malkin and McPherson, 1993a,b; Moreno et al., 2000). DLS is most commonly used prior to setting up crystallization trials in order to assess whether a sample is suitable for crystallization (Bergfors, 1999; Bolanos-Garcia et al., 1998; D'Arcy, 1994; Ferré-D'Amaré and Burley, 1994). The addition of small molecules such as cosolvents, ions, osmolytes, chaotropes, detergents, cofactors, natural substrates or analogue compounds, inhibitors, etc., may stabilize the structure or conformation of proteins and promote crystal growth (Arakawa and Timasheff, 1985b; Zulauf et al., 1989; Schein, 1990; Trakhanov and Quiocho, 1995; Bolen, 2004). DLS can be used to monitor the aggregation of proteins as a function of temperature, pH, ion strength, additives, etc. (Mikol et al., 1990; Ferré-D'Amaré and Burley, 1994; Kurganov, 2002; Saridakis et al., 2002). More recently DLS has been adapted for routine use in vapor diffusion and microbatch trials as a diagnostic tool to detect when nucleation takes place and to pin point the time at which to change the conditions in the experiment from nucleation to growth conditions. Moreover, the use of DLS has also been miniaturised and automated for application in high-throughput mode (Dierks et al., 2008).

Thus, fluorescence and light scattering are helpful to rapidly identify stabilizing conditions comprising simple additives (salts, cofactors, metals, nucleotides, etc.) to assist protein crystallization. A related application of these techniques is the generation of core metabolite libraries for proteins of unknown structure and activity. Such application seems to be robust, cost-effective, readily adapted to commercial instruments and easy to implement in individual laboratories (Poklar et al., 1997; Pantoliano et al., 2001). In fluorescence and light scattering methods the extent of temperature shift between the melting temperature (T_m) in the presence and absence of a bound ligand is assumed to be proportional to the affinity of the protein for the ligand (i.e., ligand affinity for a given binding pocket with regard to the enthalpy of unfolding, ΔH) (Matulis et al., 2005; Bullock et al., 2005). In the case of emission fluorescence the plot of intensity versus temperature has a hyperbolic shape for a two-state unfolding mechanism, which can be described by using the same equations used to describe thermal denaturation monitored by differential scanning calorimetry (Spink, 2008).

An early survey showed that approximately 86% of the proteins analyzed displayed temperature dependence (Christopher et al., 1998). More recent studies have confirmed this observation (Zhu et al., 2006). The adequate control of temperature is of particular relevance for the crystallization of membrane proteins due to the strong dependence of detergent solubility (hence, phase transitions) upon temperature (Sennoga et al., 2003; Berger et al., 2005). Because temperature deeply affects the kinetics of crystallization, the tight control of temperature in some cases permits the separation of nucleation and crystal growth stages (Penkova et al., 2002). Although temperature is one of the most critical parameters influencing protein crystal growth, its systematic manipulation to shift the equilibrium toward the formation of the crystalline phase is rarely documented. The design of a semi-automated protein crystal growth apparatus for investigation of the phase diagram and controlled crystal growth addresses this situation (Budayova-Spano et al., 2007). The set up includes a crystal growth apparatus produced in-house and a proportional-integral-derivative (PID)

electronic device to control temperature in the range from 233 to 353 K with an accuracy of 0.1 K. The apparatus allows the manipulation of small variations of temperature of the crystallization solution and to modify diverse parameters by following the crystal growth process *in situ*. However, in its current state this apparatus is not suitable for high-throughput platforms because of the fairly large amount of sample needed (i.e., the volume of the crystallization solution typically varies between 25 and 1000 ml). The recent development and commercialization of a portable thermal control platform that allows controlling and screening several temperatures simultaneously on a single microplate (Centeo Biosciences Ltd; http://www.centeo.com) has made a step forward in harnessing the full potential of temperature as a crystallization parameter. The device makes it possible to gradually change temperature, hence allowing the careful monitoring of the effect of temperature on protein crystallization and can be adapted to high-throughput platforms (Fig. 2).

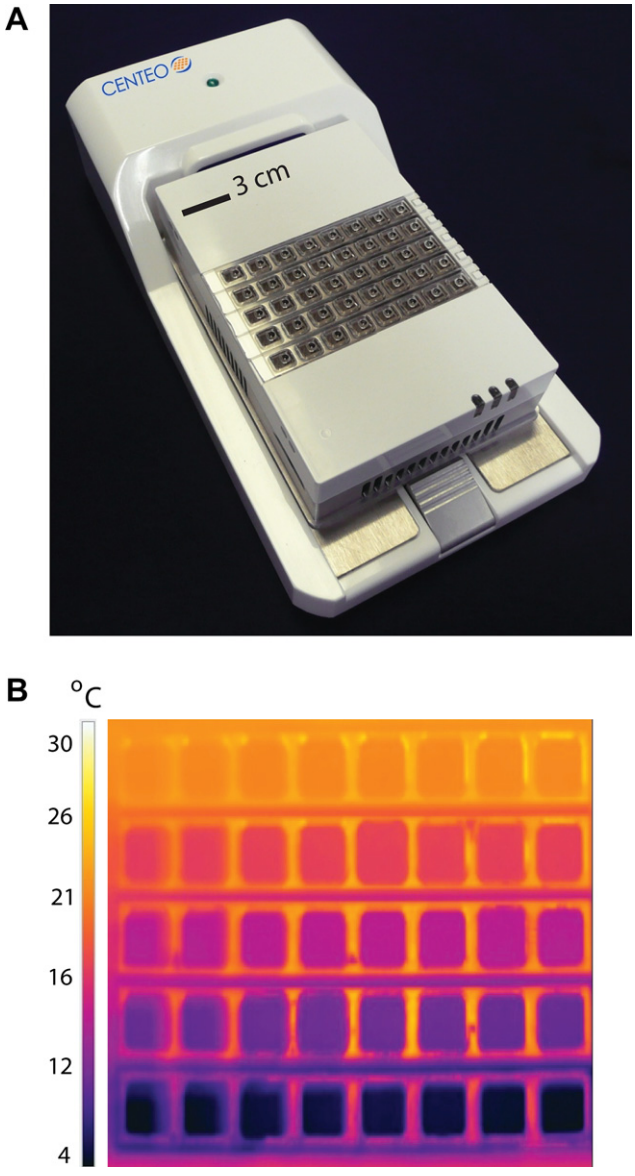

Fig. 2. T-GRID system for temperature screening. (a) The hand-held device that enables screening of different crystallization temperatures simultaneously (b) infrared scan showing that a row consisting of 8 wells can be set at one temperature whilst other rows can be set at different temperatures. This allows the simultaneous screening from 4 °C to 30 °C. This figure is used with permission from Centeo Inc.

4. Stabilizing proteins with ligands

The experimental design most commonly used for the screening of crystallization conditions is that based on sparse matrix (Jancarik and Kim, 1991) and some on incomplete factorial experiments (Carter et al., 1988; Carter and Carter, 1979; Jancarik and Kim, 1991). Although this approach is widely used in both large-scale facilities and academic laboratories, it presents important limitations such as the time involved in setting up, inspecting and optimizing crystallization trials as well as the availability of relatively large amounts of protein samples of high purity. These restrictions have prompted the search for alternative strategies such as methods that explore the stabilization of the protein structure (reviewed by Manjasetty et al., 2008). This is an aspect of outmost importance not only to improve the chances of obtaining protein crystals but also to enhance the success rates of purification of proteins that are stable and functional. The potential benefits of this approach can be illustrated by the fact that 100 out of 200 protein structures that have been determined within the Structural Genomics Consortium as of March 2006 (http://sgc.utoronto.ca/SGCWebPages/sgc-structures.php), approximately 100 could only be crystallized in the presence of a ligand, and approximately 20 of the structures were determined in the presence of ligand whose identity could not have been predicted *a priori* (Vedadi et al., 2006). Despite its advantages, the systematic study of small ligands, their classification as well as the definition of experimental conditions that enhance the stability of proteins in aqueous solutions are aspects that remain to be achieved. Nevertheless, it is not hard to envisage that in the near future the use of libraries of small-molecule compounds to prepare stable, functional proteins will become more popular in both the small laboratory and structural genomics- and proteomics-scale programs.

Amino acids and amino acid derivatives are other class of compounds that influence thermal stability and refolding of protein molecules (reviewed by Hamada et al., 2009). The effect of the addition of several amino acids (lysine, for example) and several amino acid derivatives, such as glycine ethyl ester and glycine amide, on the crystallization of equine hemoglobin and bovine pancreatic ribonuclease A has been examined by Ito et al. (2008). Thus, the addition of amino acids and amino acid derivatives to protein solutions can expand the range of experimental conditions under which single crystals of good quality may be obtained. Sometimes the simple modification of the composition of the purification buffer can improve protein stability significantly. A good example is DnaB, a protein whose DNA helicase activity (Ozaki and Katayama, 2009) had a half-life of only a few minutes at 4 °C in one particular purification buffer, but could be stabilized for hours at 60 °C after a systematic screen for optimal solution conditions (Arai et al., 1981). The use of the optimized solution conditions was essential to purify DnaB in large amounts, eventually leading to its crystallization (Arai et al., 1981).

Additives that are suitable for high-throughput platforms greatly extend the number of crystallization conditions to screen and increase the probability of identifying single crystals from the initial hits (Fig. 3). Indeed, small molecules that promote crystal growth constitute the basis of some commercial crystallization kits such as the Silver Bullets Screen and the additives kit (Hampton Research, USA; http://hamptonresearch.com; McPherson and Cudney, 2006).

5. Induction of nucleation

It is thought that most of the large protein crystals that are supposedly grown homogeneously have, in fact, been nucleated heterogeneously due to microscopic solid impurities or insoluble

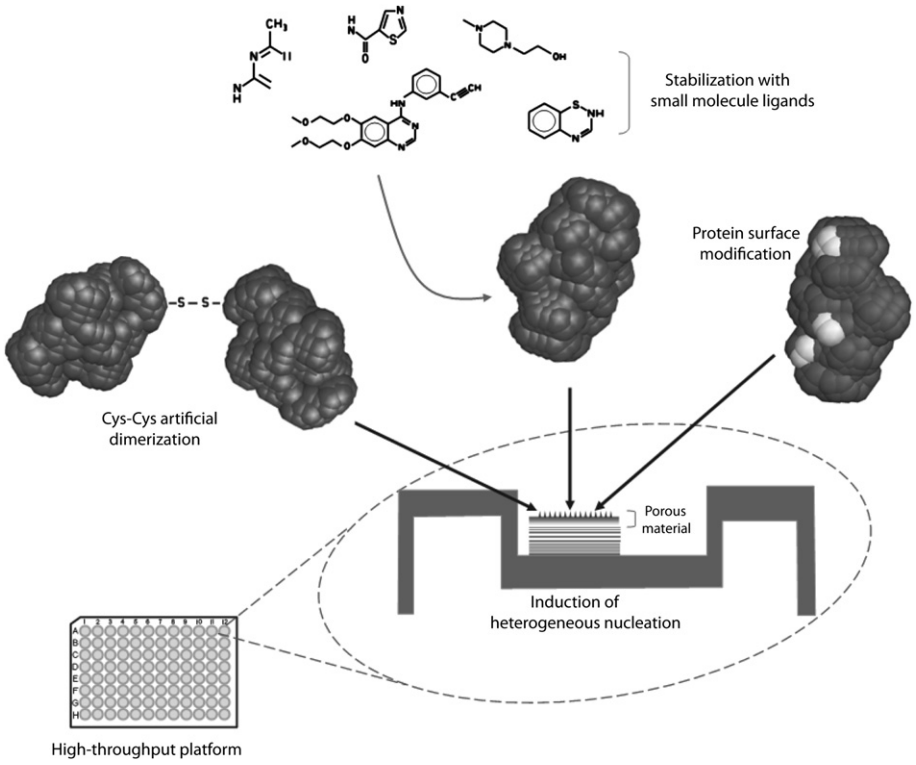

Fig. 3. Schematic illustration of a variety of ways to aid the crystallization of a protein: small molecule ligands, surface modification and/or introduction of intermolecular disulphide bridges may all help stabilize the protein which can then be exposed to screening with nucleants.

aggregates (Nanev, 2007b). It can be expected that the deliberate and selective control of the number of nuclei and the supersaturation point at which nuclei grow will lead to the formation of high quality crystals. This possibility has prompted a series of studies on the effects of the systematic introduction of heterogeneous nucleants in initial screening trials to identify a suitable crystallization condition that would have otherwise been missed (D'Arcy et al., 2003b; Thakur et al., 2007; Takehara et al., 2008). Some of the novel methods to induce heterogeneous nucleation include the use of materials such as porous silicon. Importantly, the cavities of this material are of similar sizes to those of protein molecules (Chayen et al., 2001, 2006). The cavities are expected to entrap the protein molecules, thus promoting nucleation and crystal growth (Fig. 4). Addition of fragments of this porous silicon as small as approx. 0.06 mm^2 to crystallization trials yielded crystals in 4 out of 5 of the model proteins tested and enabled crystallization at metastable conditions of a protein widely recognised as difficult to crystallize (Chayen et al., 2006). In order to gain general acceptance, heterogeneous nucleants for initial screening are expected to be applied easily within an automated, miniaturised crystallization experimental environment (Saridakis and Chayen, 2009). Initial efforts aiming to automate the delivery of nucleation-promoting substrates into the crystallization droplet either in small-scale or in high-throughput platforms have been reported in recent years (Thakur et al., 2007; D'Arcy et al., 2007).

To date the best material currently available to promote nucleation is an amorphous mesoporous bioactive bio-glass, $CaO{-}P_2O_5{-}SiO_2$, which shows a pore-size distribution in the range of 2–10 nm in diameter and a great variability of pore shapes (Chayen et al., 2006; Saridakis and Chayen, 2009). The bio-glass is simply introduced into the crystallization drops in form of grains of approximately 100 μm in diameter. This bio-glass material promotes nucleation without the need for specifically adapted conditions and irrespective of molecular weight, pH, precipitant and crystallization set up (Chayen et al., 2006). Importantly, soluble and membrane proteins have been crystallized in the presence of

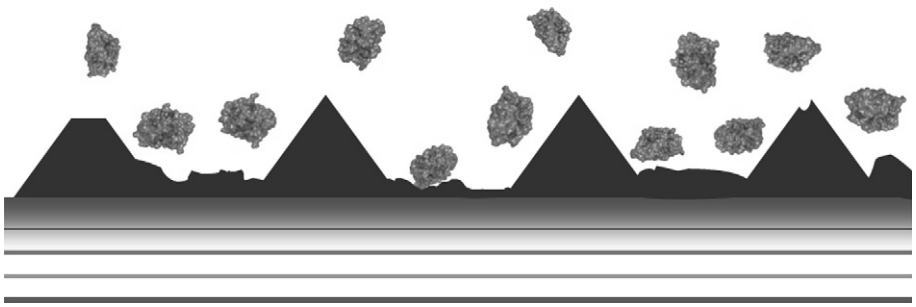

Fig. 4. Schematic illustration of protein molecules entering the pores of a nucleant. Some of the porous are expected to entrap the protein molecules, thus promoting nucleation and crystal growth.

this bio-glass material (Chayen et al., 2006). A crystallization kit consisting of the porous material $CaO-P_2O_5-SiO_2$ (Naomi's Nucleants) has been commercialized recently (Molecular Dimensions, Ltd; http://www.moleculardimensions.com/uk).

The wide range of pore sizes and shapes that are available at the surface of a disordered porous material provide a large repertoire of pores among which the given macromolecule will be likely to find the pore of adequate size and shape to nucleate (Chayen et al., 2006; Fig. 4). Furthermore, materials of diverse chemical nature with irregular, rough surface structures other than pores can be designed to act as heterogeneous nucleants. An example is semi-synthetic micromica, a material that reduces the time before crystals appeared in eight out of the ten tested proteins (Takehara et al., 2008). Furthermore, some natural substances such as cellulose and hydroxyapatite powders and pore strips have also been successfully employed as heterogeneous nucleants (Dekker et al., 2004; Thakur et al., 2007). The crystallization of a set of model proteins in pore strips shows that nucleation is comparable with that occurring in hanging-drop conditions even when using small volumes (Dekker et al., 2004). Interestingly, protein crystallization in pore strips permits the formation of different crystal forms, suggesting pore crystallization may follow a trajectory through the phase diagram that is different from that inherent to the hanging-drop method (Dekker et al., 2004).

Polypropylene or polyvinylidene porous hydrophobic membranes are other materials that promote heterogeneous nucleation of protein molecules, drastically reducing the time required to induce nucleation (Curcio et al., 2003; Di Profio et al., 2003; Di Profio et al., 2005; Zhang et al., 2008). Importantly, protein crystals that were nucleated on the surface of these membranes are able to detach from the surface and move into the solution, leaving a new nucleation site behind (Curcio et al., 2003).

Another material that acts as a protein crystallization catalyst is microporous zeolite. This is a synthetic aluminosilicate crystalline polymer with regular micropores that seems to promote crystal nucleation that is form-selective (Sugahara et al., 2008). The most successful zeolite nucleant reported by Sugahara et al. (2008) was that of a pore size of 5 Å that binds Ca^{2+} ions. The zeolite-mediated crystallization improved the crystal quality in five out of six proteins investigated and, similar to the case of pore strips, allowed the formation of novel crystal forms of better resolution. The analysis of crystal-packing revealed a layer-like structure in the crystal lattice thus supporting the notion that crystal formation occurred through a hetero-epitaxic growth mechanism (Sugahara et al., 2008).

Functionalized mica sheets and polystyrene films of different densities of amino or sulfonated groups are two other examples of materials successfully used as heterogeneous nucleating surfaces for proteins crystallization (Tosi et al., 2008). Interestingly, compared to the surface of the coverslips used in vapor diffusion methods, these surfaces often allowed the reduction of protein concentration and nucleation time required to yield crystals of model proteins (Tosi et al., 2008). Furthermore, Tang et al. (2005) have studied mica sheets silanized by 3-aminopropyl triethoxysilane as crystallization surfaces for lysozyme, trichosanthin and three other proteins of unknown crystalline structure. For the later proteins, the diffraction ability of the crystals did improve considerably.

One central aspect that needs to be addressed is the molecular understanding of the nucleation process. As reviewed by Ochi et al. (Prog. Biophys. Mol. Biol., 2009), instrumental tools such as atomic-force microscopy, scanning-electron microscopy and dual-polarization interferometry, might provide information on the detailed effects of the structure and properties of the nucleant on the attachment of target molecules. This information might in turn shed light into the definition of the molecular mechanism(s) of nucleation on nanostructures, and the effect of the patterning or roughness of their surfaces on the induction of nucleation (Rong et al., 2004; Page and Sear, 2006; Liu et al., 2007).

6. Protein modification

Molecular biology methods can be used to endow protein molecules with 'crystallizable' properties by replacement of specific amino acid residues, modification of certain sidechains and/or incorporation of posttranslational modifications. One of the first methods of chemical modification proposed for enhancing protein propensity to crystallize was the reductive methylation of lysine residues (Rayment, 1997). This chemical modification lessens the overall protein solubility due to the increased hydrophobicity of the methylated lysine sidechain, a condition that sometimes promotes the establishment of ordered crystal contacts. A recent study has shown that four out of 10 proteins previously considered as non-crystallizable became crystallizable after reductive methylation (Walter et al., 2006). Several other strategies consider the chemical modification or substitution of cysteine residues (Mi et al., 2008). One approach relies on the chemical modification of cysteine residues through reductive carboxymethylation (Eiler et al., 2001). Interestingly, as opposite to lysine modification, cysteine carboxymethylation increases the overall solubility of the protein thus preventing aggregation and unfolding (Eiler et al., 2001). In other cases, the substitution of cysteine residues might make a protein more amenable for crystallization with little effect upon protein function. One example is the DNA-binding replication terminator protein (RTP) from *Bacillus subtilis*. The structure of one RTP mutant (cysteine 110 to serine), solved to 2.5 A resolution shows no major structural perturbation due to the mutation whereas heteronuclear NMR spectroscopy revealed subtle differences in the electronic environment about the site of mutation (Vivian et al., 2003). Another example is the protein RecX from *Escherichia coli*, a protein that plays an important role in the regulation of homologous recombination of DNA. The substitution of cysteines 113 and 118 with alanine was essential to crystallize RecX (Ragone et al., 2008). As one might expect, there are counter examples where the substitution and/or chemical modification of cysteines have a profound effect on protein function, stability and/or oligomerization state, Thus, a careful analysis of the role of cysteine residues on the function of the protein of interest is required before attempting any site specific mutagenesis for the sake of its crystallization. One example of this scenario is the type II transmembrane protein and member of the TNF protein family, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) as its cysteine 230 is essential for dimer formation and the modulation of apoptotic activity (Seol and Billiar, 2000). Another case is the heterotetrameric protein kinase CK2. Many functional properties of CK2β, the regulatory subunit of CK2 that acts as a docking platform for multiple substrates (Bolanos-Garcia et al., 2006), are either suppressed or deeply altered upon chemical modification of its conserved cysteine residues. This includes the impairment of homodimerization, decreased catalytic activity of CK2 toward peptide substrates and deregulation of the stimulatory effect of polylysine (Meggio et al., 2000). One radically different strategy is the introduction of cysteine residues at solvent-exposed positions on a protein surface to form disulfide bonded artificial dimers (Banatao et al., 2006; Fig. 3). It is important that such dimers exhibit symmetry: protein molecules that are relatively symmetric seem to be more prone to crystallize (Banatao et al., 2006). One additional advantage of cysteine substitution is the possibility of creating a whole series of different constructs depending on where the attachment point is placed. A proof of principle of this method is

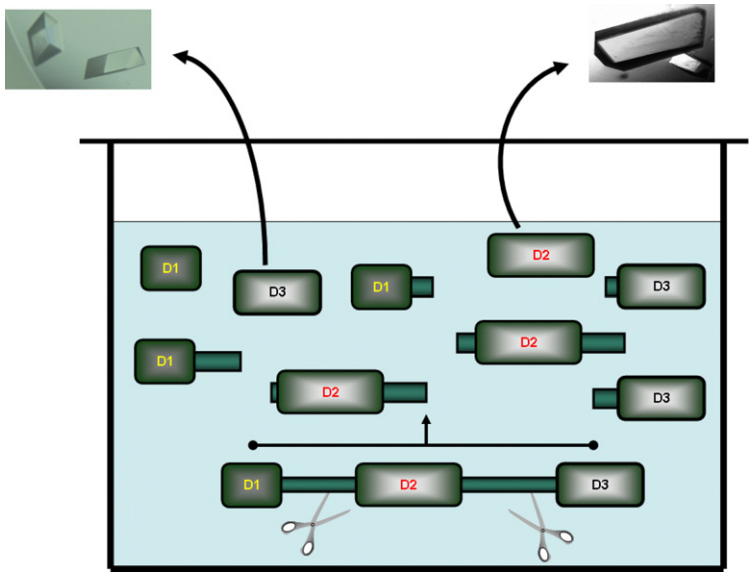

Fig. 5. Protein crystallization assisted by *in situ* proteolysis. Stable fragments of one or more protein domains (D1, D2 and D3) can gradually populate the crystallization mixture by addition of protease at nano- to micromolar concentration. The resulting fragments have a better chance to form crystals because of the expected higher stability and/or low heterogeneity of the protein solution.

the crystallization of phage T4 lysozyme, which made possible to crystallize it in six novel crystal forms from protein dimers using conventional vapor diffusion methods. The artificial cysteine dimerization approach seems particularly suitable for the crystallization of small proteins and/or isolated protein domains. In contrast, multiprotein complexes such as those mediating cell signalling are often asymmetric, pressumably to create more sensitivity and specificity in the signalling system by assembling other components into higher-order multiprotein systems (Blundell et al., 2002b). A different strategy that seems particularly suitable for soluble proteins consists of the mutation of lysine to alanine and/or glutamate to alanine (Longenecker et al., 2001; Derewenda, 2004a, b). Mutation of these two polar amino acid residues is preferred because lysines and glutamates are found predominantly on the protein's surface, with only 6% and 12%, respectively, buried (Baud and Karlin, 1999). However, as a cautionary tale it has to be said the lysine to alanine and glutamate to alanine mutations almost invariably lower the protein's solubility and at least in the case of the glutamate to alanine mutation, the substitution often results in a less stable protein (Mateja et al., 2002). It will be interesting to follow what other classes of additional posttranslational modifications are further proposed for this purpose.

In situ proteolysis is another successful method of protein modification (Fig. 5). Indeed, the application of *in situ* proteolysis doubled the success rate in protein crystallization and structure determination of one Structural Genomics effort (Dong et al., 2007). It was reported recently that the application of *in situ* proteolysis to a larger number of soluble proteins (i.e., over 270 different proteins) that had failed in the past to produce crystals suitable for structure determination increased the success rate in approximately 12% (Wernimont and Edwards, 2009). Importantly, the proteins tested in that study are derived from a wide range of species (ranging from prokaryotic organisms and protozoan parasites to yeast and humans) and exhibited functions as diverse as tyrosine kinases, GTPases and chromatin remodelling proteins. The authors commented that in some cases *in situ* proteolysis also favoured the formation of crystals of good quality, leading to crystals structures of high-resolution (Wernimont and Edwards, 2009). The screening of crystallization conditions by *in situ* proteolysis constitutes the basis of one crystallization kit currently available in the market (Jena Bioscience, GmbH; http://www.jenabioscience.com).

7. Closing remarks

It is obvious that there is no one perfect method for achieving protein crystallization and that to a great extent the field remains an empirical science. Hence, the more strategies and methods that are applied to the protein of interest the greater the chances of crystallizing it. In recent years a variety of sophisticated tools for searching, monitoring and optimizing crystallization conditions have become available, enabling crystallization to be much more rapid and efficient then previously. The combined use of conventional crystallization methods with small molecules to enhance protein stability; with diverse materials to induce heterogeneous nucleation, and/or the specific chemical modifications of protein molecules to make them more amenable to crystallize, are promising strategies to aid the production of suitable crystals for structural studies.

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