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.

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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.