Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

A review of techniques for maximizing diffraction from a protein crystal *in stilla*

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Received 6 April 2005
Accepted 10 October 2005

There are a number of published reports of techniques that may extend the diffraction limits or otherwise improve the quality of the X-ray diffraction data from a crystal. This review touches on some of these techniques to provide a way forward for the experimentalist burdened with less than ideal crystals.

1. Introduction

The process of determining the structure of a protein using X-ray diffraction necessitates the production of well ordered crystals. Sadly, the growth of a visually perfect crystal does not necessarily imply that a structure will be forthcoming, as there is limited correlation between the macroscopic beauty of a crystal and its diffraction quality (Owen & Garman, 2005). Given that crystallization is a rare event, any crystal that appears to be suitable for X-ray analysis is worth the time needed to eke out the best diffraction from it. There are many tips and techniques for improving crystal quality, ranging from annealing the crystal to re-engineering the DNA construct; this paper is intended to provide an overview of those techniques that do not require further protein production. An informal survey initiated by Anthony Duff (a.duff@usyd.edu.au) on the CCP4 bulletin board covers much of the same area. It is worthwhile looking at the results of the survey (http://www.moleculardimensions.com/uk/archive.ihtml), as they bring home the important point that all of the following techniques have a good chance of not doing anything at all to improve diffraction from your recalcitrant crystal. It has been aptly pointed out that the macromolecular crystallization literature is merely a collection of published anecdotes. Techniques such as rational mutagenesis of the construct (Dale *et al.*, 2003; Derewenda, 2004; Qiu & Janson, 2004) or modification of the expression or purification protocols (Bucher *et al.*, 2002) are not within the scope of this overview. We suggest using the phrase '*in stilla*'[1] as an encompassing term for manipulations which occur within the crystallization experiment, in the spirit of the other famous Latin '*in*'s (*e.g. in vivo*, *in vitro*, *in silico*).

A protein crystal generally yields clues about its diffraction quality only after being harvested from the crystallization experiment, introduced into a cryoprotecting solution, cooled to liquid-nitrogen temperatures and exposed to X-rays. At each stage of the process, the crystal can be damaged. For a non-diffracting crystal, the most important piece of informa-

[1] We suggest the use of the expression '*in stilla*' from the Latin *stilla* (drip; drop) for these techniques. As has been pointed out (John Sawyer, personal communication), the expression '*in lacrima*' is more suitable, as there are historical precedents for the use of the Latin word *lacrima* in classical scientific literature, but the modern association of *lacrima* with 'tears' is perhaps a little too close to home to sit altogether comfortably.

tion to discover is when the crystal stopped diffracting. Was it well ordered in the drop? Is it harmed by the cryoprotection? Or is it perhaps the cryocooling that is causing the problem?

2. Room-temperature diffraction reality check

Most crystallographers appreciate that crystals should be tested for diffraction at room temperature, but know also that the diffraction properties of a crystal after a clumsy capillary mount are not necessarily a good indication of how well that crystal could diffract under optimal conditions. The rarer room-temperature data collection becomes, the rarer becomes the skill to harvest a protein crystal into a quartz capillary. The familiar cryoloop can be used as the basis of a temporary room-temperature mounting system where a standard X-ray capillary is doped with a little mother liquor and used as a sheath over a crystal mounted in a loop (MacSweeney & D'Arcy, 2003; Skrzypczak-Jankun *et al.*, 1996). There are also some systems where a crystal can be probed by X-rays when in the growth chamber: Fluidigm (http://www.fluidigm.com; Hansen *et al.*, 2002) has a small chip suitable for mounting in the X-ray beam and there are other devices under development which enable one to probe the diffraction quality of a crystal without disturbing it (McPherson, 2000; Watanabe *et al.*, 2002). Most of these systems have limitations for data collection, as the geometry of the devices is such that a sphere of reflections would be difficult to collect. However, Ng and coworkers grew, cryoprotected, cryocooled and collected data from a crystal in a capillary using the method of counter-diffusion (Gavira *et al.*, 2002). For particularly fragile protein crystals, there have been some reported successes with hardening the crystals by limited gluteraldehyde cross-linking. This technique was first reported by Quiocho and Richards in 1964, where they used a 6% gluteraldehyde solution to stabilize ribonuclease A crystals (Quiocho & Richards, 1964). More recently, it has been shown that gluteraldehyde is sufficiently volatile that the cross-linking can be performed without having to directly add it to the drop (Lusty, 1999). In this technique, a small amount of gluteraldehyde is introduced into the reservoir of a vapor-diffusion experiment. The gluteraldehyde moves through the vapor phase and reacts with lysine residues on the surface of the crystal. The amounts of gluteraldehyde needed to induce hardening of the crystal are very small; too much cross-linking and you end up with an extremely robust yellow crystal that is very unlikely to diffract X-rays at all (Quiocho & Richards, 1964). Although the early studies used gluteraldehyde concentrations of up to 25%, less is probably more, and it has been suggested that adding gluteraldehyde to a level of 0.001% final concentration in the reservoir is a reasonable starting point. Certainly, there are published reports of effective crystal hardening at the 0.01% gluteraldehyde level (Gray *et al.*, 1998).

3. All crystals are not equal

A crystallization experiment usually consists of setting up more than one drop: in the optimization stage of crystallization trials, many drops may be set up with similar, if not identical, conditions. It is unwise to assume that all crystals grown under the same conditions or indeed in the same drop are equivalent; it is not uncommon that different crystals in the same drop may have different space groups (Pohl *et al.*, 1998). This suggests that if one crystal does not diffract well, another grown under similar conditions may. Over 400 crystals were screened of the 1000 crystals cryocooled during the elucidation of the structure of the small subunit of the ribosome (Brodersen *et al.*, 2002). It is likely that one might find a crystal with lower mosaicity or higher resolution by screening; it is not so likely that if 99 crystals of the same protein grown under the same conditions show no diffraction that the 100th crystal will.

4. Crystal manipulations

4.1. Crystal annealing

A prize-winning poster from the 1997 American Crystallographic Association (ACA) meeting showed that a cryocooled crystal could be removed from the cold stream, returned to a drop of cryoprotecting mother liquor at room temperature, then re-looped and re-cooled (Harp *et al.*, 1998). This 'crystal annealing' did not kill the crystal; in some cases it even improved diffraction by either removing artifacts of a poor freeze (ice rings, for example) or by reducing the mosaicity, increasing the resolution or both. These results were greeted with some skepticism, but have been tried with varying success since then (Hanson *et al.*, 2003). There are two main approaches to crystal annealing: the simplest is to block the cold stream temporarily so that the crystal thaws and then to unblock the cold stream so that the loop-mounted crystal refreezes. This thaw/freeze cycle can be continued until the crystal stops improving (Samygina *et al.*, 2000; Yeh & Hol, 1998). The second approach involves removing the crystal from the mounting loop into a drop of cryoprotecting mother liquor, then waiting for some time (up to 3 min), re-looping and re-cooling (Harp *et al.*, 1998). This method has the advantage that one can change the cryoprotecting mother liquor, but has the disadvantage that the crystal will be in a different orientation in the X-ray beam for subsequent exposures, making direct comparison of the quality of the crystal before and after the process more difficult. Why crystal annealing works is unclear, but it seems that it works best in cases where the initial cryoprotection and/or flash-cooling was suboptimal (Juers & Matthews, 2004).

4.2. Crystal dehydration

There is some confusion in the literature about how to define 'dehydration'. Sometimes 'dehydration' is used only if the manipulations *in stilla* result in unit-cell shrinkage (Esnouf *et al.*, 1998); the more general usage seems to include cases where the crystal is soaked in an aliquot of mother liquor which is then allowed to evaporate to some extent (Abergel, 2004). In a recent paper (Abergel, 2004), it was reported that diffraction from a number of crystals was maximized if the

technique of crystal annealing was coupled with dehydration. The study was prompted by the observation that the gradual dehydration of a crystal droplet over the course of years in a plastic crystallization tray had improved the diffraction from a crystal from the dehydrated drop. A moderate increase in crystal quality through dehydration had been noted before (Esnouf *et al.*, 1998) and indeed there is a commercially available device that allows one to examine the diffraction properties of a crystal whilst manipulating the relative humidity of the crystal (Kiefersauer *et al.*, 2000). However, the coupling of initial bad cryocooling with subsequent room-temperature rapid (minutes to hours) evaporation produced some stunning increases in useful information from the treated crystals. The most widely used method for dehydrating crystals appears to be to relocate a crystal in the crystallization droplet over a reservoir with a higher percentage of precipitant (Heras *et al.*, 2003). However, a crude method of simply waving the loop-mounted crystal around a bit before flash-cooling has also (apocryphally) been reported to work. In the 'wave the crystal' method, one wants to wait no longer than the appearance of cloudiness in the harvesting loop, as this suggests that the saturation point of the salts in the crystallization solution has been reached (Samygina *et al.*, 2000). A recent review of dehydration by Heras & Martin (2005) provides a careful literature search and describes in some depth the experimental details of various dehydration protocols.

4.3. Crystal soaking

The introduction of higher concentrations of precipitating agent may increase the quality of diffraction from a crystal, without the evaporation or decrease in unit-cell parameters that are the trademarks of dehydration. This post-crystallization soaking may simply be in the same or similar conditions under which the crystal was initially grown, or may require the introduction of other chemicals into the crystal to improve its diffraction quality (Petock *et al.*, 2001). The main difficulty here is that one cannot rely on controlled dehydration to produce the changed mother liquor; the 'other chemical' has to be added in such a way that the addition process does not destroy the crystal before the 'other chemical' can improve it. Post-crystallization soaking has also been used to minimize the non-isomorphism observed between native and derivative crystals of the 30S subunit of the ribosome by soaking the 'native' crystals in a light-atom analogue of the heavy-metal derivative (Brodersen *et al.*, 2002).

4.4. Crystal tempering

The soaking and dehydration improvements seen are believably a function of rearrangements of parts of the crystal: either mosaic blocks aligning better with each other or the jiggling of individual molecules into a more ordered form. Wim Hol (personal communication) has suggested that a mechanism for speeding up these rearrangements might be to temper the crystals: that is, to heat up the crystal, still in its crystallization droplet, and then to let the whole system cool back to the original temperature very slowly. How much one heats the crystal probably is dependent on how confident one is feeling at the time: the range of 10–40 K has been suggested. Although this procedure is reasonably widely known, it is hard to find literature references where this method has been applied.

4.5. Cryoprotection

Many papers have been written on crystal cooling and the cryoprotection that is required for this to work (see Garman, 1999, 2003, as entry points into the literature). The take-home message is that 20% glycerol is not the only cryoprotectant available: high concentrations of salt often cryoprotect (Holyoak *et al.*, 2003; Rubinson *et al.*, 2000), as do sugars and low-molecular-weight polyethylene glycols. Cryoprotecting a crystal requires optimization in much the same way that crystal growth does: one needs to think of both the type of cryoprotectant, the concentration of the cryoprotectant and how to introduce it into the crystal. Perhaps the most robust way to do this is by growing the crystal under cryoprotecting conditions. This is a great strategy, but one that is hard to apply retrospectively. It is important to notice that the concentration of cryoprotectant required to ensure the formation of an amorphous glass on flash-cooling is a lower limit to what may be appropriate for producing the best diffraction from the cooled crystal (Mitchell & Garman, 1994). Furthermore, recent work by the Schall group has shown that the concentration of cryoprotectant required for a successful amorphous freeze is highly dependent on the size of the crystal (Chinte *et al.*, 2005). For systems that tolerate high levels of cryoprotection poorly, choosing a small crystal may solve the problem.

5. Protein manipulations

5.1. Protein cleanup and stability enhancement

There are ways of preparing a protein sample that may aid its crystallization. These fall into two classes: techniques that promote the stability or homogeneity of the sample and those that alter the surface properties of the protein. Perhaps the easiest is simply to centrifuge or filter the protein before setup, in order to spin down large aggregates and dust that may contribute to crystal imperfection or overnucleation. Another simple treatment is to expose the protein sample to a moderate (15–20 K) increase in temperature for a short while (5–15 min) and to then centrifuge the sample. The rationale is that the heat forces the denaturation and precipitation of marginally stable or improperly folded protein molecules, which can then be removed from the system by centrifugation, thus leaving the sample more homogeneous (Pusey *et al.*, 2005). Jancarik *et al.* (2004) have suggested that screening buffers and pH that enhance the solubility and monodispersity of a protein sample can improve crystallization, both in terms of number of hits and crystal quality. Adding ligands may enhance the stability of the protein and also aid in the crystallization process. Technologies such as Thermofluor™, in

which the thermal denaturation curve of a protein is followed using a fluorescent dye, can be used not only to gauge the extent of protein stabilization on ligand binding, but also to find small-molecule binders in cases where the protein function is unknown (Carver *et al.*, 2005).

5.2. Side-chain modification

Heavy metals may fall into both classes as they may both stabilize the protein and alter its surface properties (Pakhomova *et al.*, 2000). These compounds tend to bind to exposed cysteine or acid side chains. There are many heavy-atom compounds that one could introduce into a protein sample and most experimentalists do not have the resources to test them all systematically. Gel electrophoresis has been used (Boggon & Shapiro, 2000) to select heavy-atom compounds that may be useful in phasing diffraction data: the same logic applies as well to the idea of finding compounds which might bind selectively to a protein to alter its surface properties. There are many reagents that will specifically modify particular side chains (Lundblad, 2004); given the wealth of possibilities, it is perhaps surprising that only the reductive alkylation of lysines appears regularly in the crystallization literature (Rayment, 1997; Schubot & Waugh, 2004). A simple chemical modification to free cysteines is quite likely to occur just by adding the reductants β-mercaptoethanol or dithiothreitol (Begg & Speicher, 1999).

5.3. Proteolysis

More dramatic modification can occur through the action of proteases. Traditionally, a protein is tested for a stable fragment by running time-course experiments with small amounts of different proteases such as trypsin, chymotrypsin, V8 protease, carboxypeptidase and aminopeptidase. If a stable fragment is observed then either the protein sample is proteolysed and repurified to make that fragment or alternatively (and more commonly) a new construct is made which encodes that stable fragment. McPherson and coworkers demonstrated that this search can be conducted *in stilla* (McPherson *et al.*, 2004) by simply adding a trace amount of protease to the crystallization trials. The authors argue that this technique is particularly suited for those cases where it takes months for crystals to appear, in which the rate-limiting factor may well be the production of the correct fragment for crystallization.

6. Conclusions

There are a number of manipulations that are available to the experimentalist with poorly diffracting crystals. However, there are no systematic studies that have mapped particular problems to particular solutions and certainly no guarantees are issued with any of these methods. With no other game plan available, it is reasonable to start off with the methods that take up the least time and effort (crystal annealing, crystal tempering) before moving on to techniques that demand a significant investment of resources (Thermofluor™ investigation of potential ligands). Once again, the need for reproduciblity in the crystallization is paramount, as it is unlikely that any one crystal would survive the gamut of all the possible perturbations to which it could be subjected.

I am very grateful to the organisers of the 25th CCP4 workshop for inviting me to speak on this interesting topic. I thank Dr Tom Peat for his patient discussions and Dr Lindsay Sawyer and Dr John Sawyer for encouraging my Latin diversions.

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