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Perspectives on protein crystallisation

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ABSTRACT

This final part on 'perspectives' is focused on new strategies that can be used to crystallise proteins and improve the crystal quality of macromolecular complexes using any of the methods reviewed in this focused issue. Some advantages and disadvantages, limitations, and plausible applications to high-resolution X-ray crystallography are discussed.

1. Introduction

Most of the biological projects based on crystallography lack suitable crystals for high-resolution X-ray crystallographic investigations. Several scenarios exist in which obtaining the adequate crystal is particularly difficult; for instance, some apo-forms are difficult to crystallise, whereas crystallisation of the protein–ligand complex is less difficult to achieve. There are some cases where crystals are sensitive to changes in temperature so that they will be damaged by freezing. Sometimes, isolated proteins, protein–protein or protein–DNA/RNA complexes disassemble quickly before forming a high quality single crystal, if diffusion-control is selected as the crystal growth method. In these particular cases, it is difficult to obtain the 3D structure at high resolution due to the kinetic factors involved in the crystallisation process. In specific cases, the presence of metal ions is strictly necessary to stabilise the structures and to facilitate crystallisation, whereas for others removal of these ions inhibits the crystallisation process (Patel et al., 2002). Indeed, the use of divalent cations in protein crystallisation is sometimes key to obtaining high quality single protein crystals.

As already described in this focused issue, many methods of crystal growth exist. However, additional research is needed in terms of exploring and understanding the effects of physical and chemical properties of macromolecular solutions and protein–protein or protein–ligand interactions. Understanding the nucleation and crystallisation processes as well as controlling the size and quality of macromolecular crystals of different proteins for structural investigations is still a challenge in many laboratories worldwide. The concept of nucleation and its connection to crystal growth has to be understood in order to select the appropriate crystallisation method to separate both processes when necessary (Chayen, 2006). The analysis of nucleation applied to protein crystal growth from the theoretical point of view has been reviewed elsewhere (Nanev, 2007). Protein solubility and temperature dependency have proven to be useful tools when growing crystals suitable for X-ray analysis (Astier and Veesler, 2008). Finally, the mechanisms of protein crystal growth are not usually investigated in structural biology projects; however, they can give rise to valuable information about the supersaturation conditions as well as on crystal quality improvement.

The mechanisms of crystal growth are particularly important to understand the history of the crystallisation process. Scanning electron microscopy, as well as atomic force microscopy, pioneered efforts in the investigation of crystal growth mechanisms (Durbin and Carlson, 1992). These methods focused on examining the surfaces of lysozyme crystals as well as of model proteins and virus particles, and revealed new mechanisms of crystal growth (Hiroyuki, 1996; Malkin et al., 1996; Walters et al., 1997; Reviakine et al., 1998; Li et al., 1999; Kim et al., 2000; McPherson et al., 2000; Wheeler and Sikes, 2000; Yau et al., 2000a,b; Dufrene, 2001; Plomp

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et al., 2003). More detailed reviews, which include thorough discussion of the effects of several biochemical and biophysical parameters on crystal growth, have been published by McPherson (2001) and McPherson et al. (2003). The relationship between atomic force microscopy and X-ray diffraction studies has been published testing proteins and virus crystals (Malkin and Thorne, 2004). A comparison of different experimental techniques for the measurement of crystal growth kinetics was recently published (Van Driessche et al., 2008). Atomic force microscopy revealed that crystal growth occurred by a lattice defect mechanism at low supersaturation through 2-dimensional nucleation (layer-by-layer or spiral growth due to the existence of dislocations). However, at intermediate supersaturation the observed mechanism is 3-dimensional nucleation due to island growth. The last mechanism by adhesion-growth (called also normal-growth) is usually obtained at a very high supersaturation. More recently, atomic force microscopy (AFM) has become a common tool in the biophysical studies of proteins. This is mainly due to its ability to characterise tertiary and quaternary structures, forces driving folding–unfolding processes, and secondary structure elements in their native environments (Yang et al., 1993; Hallett et al., 1995; Pang et al., 1997; Chittur, 1998; Cao et al., 2001; Huang et al., 2002; Torres et al., 2003; Silva, 2005; Tulpar et al., 2005). It is important to remark that in addition to AFM, other techniques suitable for the surface characterisation of protein crystals include interferometry methods, in particular the two beam and improved phase shifting methods (McPherson et al., 1995; Gliko et al., 2002; Yin et al., 2008). For some time the main problem was fixing the crystals prior to scanning the soft protein surfaces by means of AFM-EC (Atomic Force Microscopy-Electrochemistry) methods. This problem has been overcome by using polypyrrole films to immobilize protein crystals on graphite (Hernández-Pérez et al., 2002). Indeed, this technique has been applied to fix cytochrome *c* crystals on Indium Tin Oxide (ITO) electrodes for AFM-EC investigations to measure the electron transfer properties and for the development of biosensors (Acosta et al., 2007).

From the crystal growth standpoint, it is possible to use biophysical methods, like atomic force microscopy, scanning electron microscopy, confocal microscopy, and interferometry, to show the areas in which different crystal growth mechanisms are present. Fig. 1 shows the theoretical areas (in a plot of protein solubility *versus* temperature), where single crystals can be obtained as well as the implicit mechanisms of crystal growth. Definition of these areas provides an explanation as to why a crystal that grows at high supersaturation will diffract the X-ray radiation

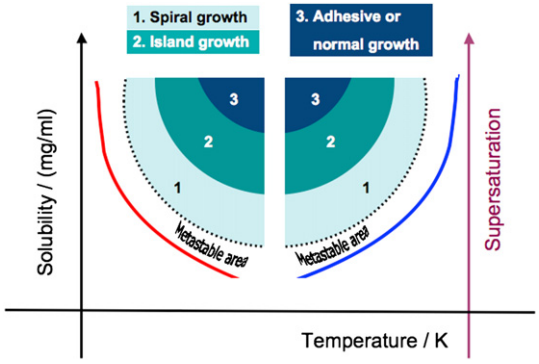

Fig. 1. Conceptual plot of mechanisms of protein crystal growth and its dependency upon temperature (positive in blue colour, negative in red). Adhesive crystal growth takes place at very high supersaturation, whereas island growth and spiral growth happen at intermediate and lower supersaturation values, respectively (Gutiérrez-Quezada et al., 2009).

in a poorly constructive fashion (due to adhesive or normal crystal growth mechanism) compared to those grown at intermediate and low supersaturation values. The reason for this improvement in crystal quality is that the latter crystals usually grow by one of two possible mechanisms: island growth or spiral growth. Fig. 1 also shows that varying temperature, as the crystallising parameter, is the best way to produce high quality crystals. These mechanisms of crystal growth also describe the formation of high quality single crystals by microseeding techniques. For instance, if one protein nucleates at high supersaturation, the crystal growth cell will be filled with small crystals of irregular shape. Most of the time, these crystals will show poor X-ray diffraction due to the adhesive crystal growth mechanism that characterises the process at the beginning of the nucleation phenomenon.

It can be inferred from Fig. 1 that adhesive crystal growth occurs at the beginning of the process due to the very high supersaturation. However, if supersaturation decreases slowly due to mass consumption, then the crystal will continue to grow by island growth ending until spiral growth mechanisms. In general, this method to obtain a crystal at high supersaturation is not suitable for X-ray diffraction due to its poor internal crystalline order. However, these types of crystalline aggregates (microcrystals) can be used as source of seeds to be added to pre-equilibrated droplets by means of the automated microseeding matrix screening for high-throughput methods (D'Arcy et al., 2007), or by microseeding techniques, as pointed out by Stura and Wilson in 1991. A comprehensive review of seeding techniques has been published by Terese Bergfors (2003).

Nowadays, we can combine several crystal growth methods to obtain high quality crystals. This is possible because in many cases the amount of protein available is not a limiting step, particularly when it can be over-expressed in a host organism. It is clear that there is no such a thing as a universal crystallisation method. However, some useful strategies have been developed to succeed in obtaining better crystals that allow the structure characterisation of macromolecular assemblies, protein–protein and/or protein–DNA complexes. The use of high-throughput methods is recommended as the first strategy for the crystallisation of a new protein. Most of the robots available on the market are very practical for identifying preliminary crystallisation conditions. Once crystallisation conditions are found and have been optimized, crystals can be grown by means of any of the crystal growth methods already mentioned in this issue. The final step, previous to the X-ray data collection, is to know how to manipulate or to prepare these crystals for a proper and successful data collection, either in synchrotron or in house diffractometers. In many cases crystal quality depends on the size of the protein crystals. However, occasionally big crystals (having high content of water molecules) show low resolution, which makes them unsuitable for a proper crystallographic research. Some practical techniques in capillaries used to replace structural-water molecules by organic solvents or polymers (ethylene glycol, glycerol, polyethylene glycols, 2-methyl-2,4-pentanediol, etc.) might be necessary to improve the quality of single crystals (Warkentin et al., 2008).

2. Crystal quality improvement and post-treatment of crystals for X-ray crystallography

Because cryocooling and cryo-protection are essential stages in crystal treatment and/or to improve crystal quality, the need to identify routes for optimization of generally applicable cryotechniques is becoming more pressing (Garman and Doublié, 2003). This includes macromolecular crystal annealing, flash cooling and dehydration procedures. Figs. 2–4 show a summary of the basic procedures of post-crystallisation-treatment that allow

improvement of crystal quality using different strategies (Heras and Martin, 2005). The first approach is related to the improvement of the crystal size. Fig. 2 shows the way to increase this size from small crystals grown in droplets. This is performed by transporting the coverslip with the protein/precipitant droplet that contains the tiny crystals to a series of concentrations of the reservoir (Saridakis and Chayen, 2003). The idea of this method is based on the typical solubility diagram that helps one to understand the crystallisation process for biological macromolecules (shown on the left side of Fig. 2); the nucleation step is usually reached at high supersaturation whilst crystal growth is obtained at lower supersaturation. This strategy demonstrates that nucleation can be separated from crystal growth (which is not an easy process to deal with), by using the conventional hanging-drop (or sitting-drop) vapour diffusion methods. This strategy has been successfully applied for the crystallisation of xylanase in the presence and absence of the solubility plot using microfluidics and high-throughput methods (Shim et al., 2007).

A summary and practical collection of the basic protocols that can be used for crystal growth using the classic method of hanging-drop in vapour diffusion are shown in Fig. 3. These can be extended to crystallisation in capillary tubes applying the counter-diffusion techniques using the Granada Crystallisation Box (García-Ruiz et al., 2002), as shown in Fig. 4. In all cases related to capillary tubes, the lower part of the capillary tube is the most appropriate area to harvest the best quality crystals for X-ray diffraction. The theory and details about how these counter-diffusion methods work along the crystallisation process, as well as the transport properties and applications, have been published elsewhere (García-Ruiz, 2003).

Although crystal growth in capillary tubes and in gels often produces high quality single crystals, sometimes is necessary to apply the same concepts of dehydration of the aforementioned protocols used for droplets (Fig. 3) to improvement of crystal quality in order to answer biological questions based on structure information. The idea described in Fig. 4 is based on a case study where a protein–protein complex was grown in capillary tubes by using counter-diffusion methods. However, the diffraction limit was not as high as expected compared to the vapour diffusion or counter-diffusion methods (García-Ruiz, 2003). The procedures described in Fig. 4 can be easily adapted to any new protein or macromolecular complex that needs improvement of crystal quality. The diffusion of the precipitating agent in either sequential increment of its concentration or at a high initial concentration

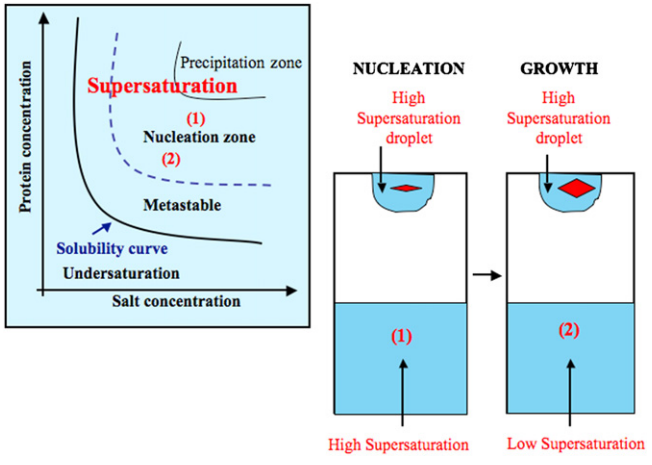

Fig. 2. Crystal growth of any biological macromolecule could be performed travelling from point (1) in the plot of the left-hand side to point number (2) for growing large crystals suitable for X-ray diffraction (adapted from Heras and Martin, 2005).

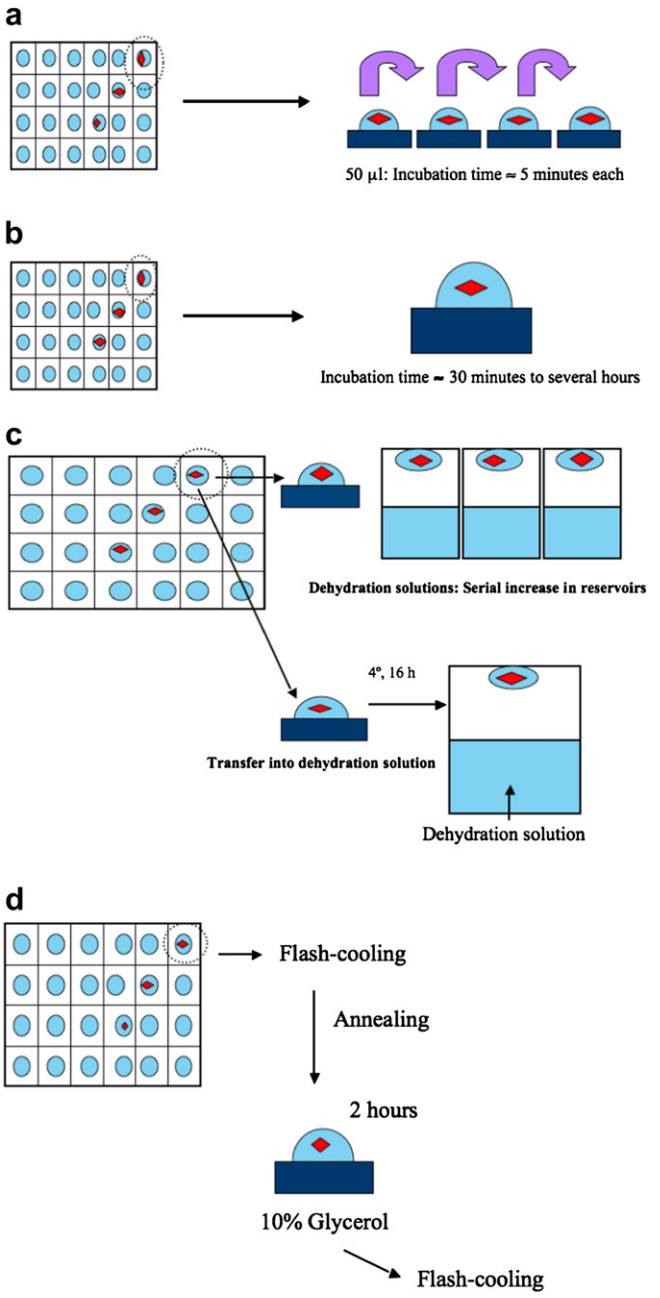

Fig. 3. This shows the three basic protocols for dehydration of protein crystals in droplets as well as combination of dehydration and annealing, (a) dehydration protocol by using serial increments of the precipitating agent, (b) protocol for incubation in the solution and evaporation in air at room temperature, (c) serial increments of the precipitating agent and transfer to a close reservoir, (d) combining macromolecular annealing, flash cooling, and dehydration methods (adapted from Heras and Martin, 2005 [doi:10.1107/S0907444905019451] with permission of the IUCr journals).

will not damage the crystals by changes in the osmotic pressure inside the crystal (Fig. 4a). In many cases the addition of a cryo-protectant along with the precipitating agent has been very effective (Fig. 4b). Although the extraction of crystals from the capillary tubes requires some expertise, by using a homemade rubber pipe and blowing away from one of the open ends of the capillary tube the crystals can be removed and deposited into a two-well or nine-well glass plate. Traces of gel should be completely removed from the crystals to warrant a higher diffraction and a good data collection.

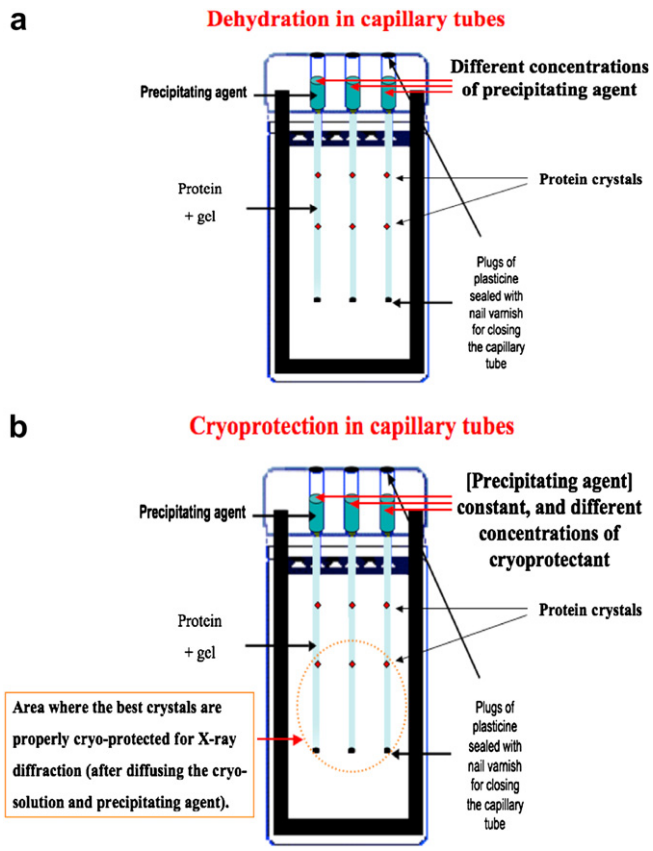

Fig. 4. Two basic dehydration protocols to enhance the crystal quality (a) by increasing the concentration of the precipitating agent, after obtaining crystals, and (b) keeping constant the concentration of the precipitant and increasing the concentration of the cryoprotectant.

3. Crystallisation of macromolecular complexes and crystal quality requirements

Thanks to the contribution from individual or collective laboratories and structural genomics' projects, over 61,000 biomacromolecular structures, among them 34,000 unique structures from non-redundant sequences are deposited in the Protein Data Bank (PDB: http://www.rcsb.org/pdb/home/home.do). Furthermore, to date the number of structures of heterogeneous macromolecular complexes, which have unique PDB ids, available in Protein Quaternary Structure Server (PQS: http://www.ebi.ac.uk/pdbe/pqs/) is approximately 8800. A more detailed analysis of the data available in PQS shows that the number of heterogeneous molecular complexes forming higher order than dimeric is about 5600, which is about 9% of the total number of structures.

Combined with low-resolution structure determination methods, e.g. electron microscopy (Rossmann et al., 2005), and small angle X-ray and neutron scattering (Petoukhov and Svergun, 2007; Putnam et al., 2007), it is possible to model biological macromolecules and their assemblies. In addition to experimental determination of protein structures, recent developments in computational modelling make it possible to predict structures of macromolecules and macromolecular complexes, which are challenging to observe by high-resolution X-ray crystallography. One good example of the benefits of this approach is the structure model of nuclear core complexes, which combined various experimental and computational methods to build a model of the 50 MDa nuclear core complex (Alber et al., 2007a,b). Those new techniques gave insight into biological questions, which had been unresolved for so many years. The techniques bridged between visible light and atomic resolutions of images of biological samples.

Nowadays, in order to know the molecular mechanisms of diseases and to design drugs, it is very useful to have direct atomic level information derived from X-ray crystallography and NMR. Although initially they represented big challenges, large macromolecular assemblies and some single macromolecules of low complexity have been crystallised and their structures have been solved. Examples of those assemblies are Ribosomes (Yonath et al., 1980; Trakhanov et al., 1987; Glotz et al., 1987; Ban et al., 1998), RNA polymerase II (Fu et al., 1999) and Fatty Acid Synthases (Jenni et al., 2006; Leibundgut et al., 2007; Lomakin et al., 2007) to name a few. Even though crystals of macromolecular complexes or single biomolecules can be obtained and the crystallisation conditions optimized, such crystals may not diffract well. In these cases, post-crystallisation-treatment of crystals, e.g. dehydration, cross-linking, is sometimes helpful to obtain well-diffracting crystals (Heras and Martin, 2005; Newman, 2006). One of the reasons to be low-resolution protein crystals is due to the high solvent content that is present in the crystal (Kantardjieff and Rupp, 2003). The analysis of the relationship between the maximum resolution and solvent content of crystals of macromolecules indicates that the lower the solvent content the higher the resolution is. Because it is likely that higher symmetries have higher solvent contents (Chruszcz et al., 2008), if macromolecules can form crystals in other space groups, those crystals may have lower solvent content and result in higher resolution of electron density maps. However, even if the solvent content is very high, it is not impossible to get medium to high-resolution structure information (Kantardjieff and Rupp, 2003). This means that we can definitely improve the quality of diffraction without exploring other crystal forms. Because protein crystals have several sources of imperfection (Malkin and Thorne, 2004), it is possible to improve the quality of crystals by overcoming those problems.

The relationship between imperfection and quality of crystals, in terms of both pure crystal growth and structural biology, has been reviewed by Malkin and Thorne (2004). Their analysis shows that in general terms there are two types of imperfections, which affect mainly B-factor and mosaicity. The former case includes diverse conformational variations and orientations. Sometimes protein conformation heterogeneity can be reduced by adding ligands or forming complexes with other macromolecules. Alternatively, changing surface properties of the molecules by chemical modifications or site-specific mutagenesis might reduce the variation of orientation.

Since lower mosaicity gives a higher intensity of diffraction spots (Helliwell, 2005), better quality of X-ray diffraction data can be obtained. Incorporation of impurities is one of reasons of the high mosaic spread of crystals (Malkin and Thorne, 2004). For instance, impurities in crystallisation solutions can increase the number of twinned crystals and alter the solubility of target proteins (Lorber et al., 1993). As is seen in an example of lysozyme using X-ray topography analysis, 5% of the dimers among the monomers of lysozyme increased full-width half-maximum of rocking curve of crystals (Lorber et al., 1993).

Incorporation of impurities in crystals should be minimized in the crystallisation process. Crystal growth in gel media (as shown in the previous review) reduces the incorporation of impurities and, as a result, improves quality of crystals (Chernov, 2001). Since contaminants can also be removed by sequential crystallisation (Malkin and Thorne, 2004), this approach is an alternative route to improve the quality of biological macromolecular crystals. Even if target molecules to be crystallised are pure, they might partially denature or aggregate while their crystals are growing and

eventually absorbed onto the surface, thus preventing further crystal growth (Malkin et al., 1999). Seeding of crystals in trials containing fresh protein sample may improve quality of crystals. However, because impurities cause the cessation of crystal growth (Malkin et al., 1999; Malkin and Thorne, 2004), the surface of crystals should be washed in crystallisation solutions before seeding. Incorporation of microcrystals onto the surface of a bigger crystal has the same effect as the incorporation of impurities and usually results in a reduced diffraction power (Malkin et al., 1999; Carotenuto et al., 2002; Malkin and Thorne, 2004). This problem can be avoided by lowering supersaturation (Malkin and Thorne, 2004). To achieve this, counter diffusive crystallisation in capillary tubes by using agarose gels is recommended (García-Ruiz et al., 2002). This is because this crystallisation method allows the formation of a supersaturation gradient along the capillary tube (García-Ruiz et al., 2002), reducing the chances of incorporating microcrystals onto the surface of growing single crystals.

Even crystals that have not incorporated impurities may still exhibit defects like vacancies and dislocations, with the resulting decrease of their diffraction limit. It might be also possible to minimise this effect by reducing convection. Crystallisation under microgravity conditions is ideal to eliminate convection (Carotenuto et al., 2002). However, this technique is expensive and not easily available. A similar reduction of convection can be achieved by combining gels and strong magnetic fields (e.g. about 7 and 10 Tesla). The latter can be performed with conventional NMR (Lin et al., 2000; Qi et al., 2001; Moreno et al., 2007; Gavira and García-Ruiz, 2009). Separation of nucleation and crystal growth processes has been shown to improve the quality of biomacromolecular crystals (Saridakis et al., 2002). Thus, this approach is worth trying to obtain well-diffracting crystals.

Cryo-protection of crystals is an important step to get good diffraction data even when regular, single crystals had been obtained. Various classes of cryo-protectants, e.g. ethylene glycol, glycerol, MPD, etc., should be screened to minimise crystal damage. Combination of cryo-protectants and oils has been reported to give higher resolution than cryo-protectants alone (Kwong and Liu, 1999). To reduce osmotic pressure, it is recommended to increase the concentration of cryo-protectants gradually (Heras and Martin, 2005; Rodgers, 1997) or to use dialysis method (Fernandez et al., 2000). If crystals are grown in the counter-diffusion method in gel and capillaries (Jaramillo et al., 2001), it is possible to increase the concentration of cryo-protectants simply by gradually replacing crystallisation solutions (as shown in Fig. 4). In this case, X-ray diffraction data can be collected without taking out crystals from capillaries (Jaramillo et al., 2001; Parkin and Hope, 1998). When crystals are fished in nylon loops, extra care must be given to avoid excessive mechanical stress. During flash-freezing the crystal should hang in the middle of the cryo-loop. If the cryo-loop is too large, the background might increase (Parkin and Hope, 1998). Therefore, cryo-loops should be carefully selected and perfectly cleaned before fishing crystals (Yoshizaki et al., 2006).

3.1. Case study: *Saccharomyces cerevisiae* DNA-repair proteins Lif1p–Lig4p

Since each crystal is expected to present particular problems, it would be ideal to know what causes particular crystal not to diffract well and the first step is to improve its quality.

We now describe an example where counter-diffusion methods, *in situ* crystal dehydration, and *in situ* cryo-protection procedures are applied to a novel protein, a DNA-repair protein (Lif1p–Lig4p) in order to improve the crystal quality. DNA-repair refers to a collection of processes by which a cell identifies and corrects damage to the DNA molecules that encode its genome. Lif1p–Lig4p is a macromolecular complex related to DNA-repair in yeast used as a case study to implement all these procedures to the crystal quality enhancement (T. Ochi, unpublished results).

From our experimental results using these approaches 8 out of 23 crystals (≈35%) diffracted better than 3.9 Å, the resolution reported in earlier studies (Dore (2006) and Dore et al., 2006) (Fig. 5a and b). After indexing and scaling diffraction images from the best crystals of condition B by Denzo and Scalepack (Otwinowski and Minor, 1997), the resolution limit was determined to be 3.5 Å. In order to compare diffraction data statistics of crystals grown in conditions A and B, the 3.5 Å data set was processed at the resolution range of 50–3.9 Å. Compared to crystals in condition A, the unit cell dimensions a and b of crystals of condition B shrank 0.76 Å whereas c extended 0.36 Å. As a result, the unit cell volume of crystals of condition B is reduced by 0.2%. Also, the mosaicity of crystals of condition B is reduced 0.16° compared to that of condition A, while $I/\sigma(I)$ of condition B was over twice larger than that of condition A. Overall, the quality of crystals of condition B was improved with the counter-diffusion gel capillary method (Table 1).

The fact that more than one crystal diffracted better than the maximum resolution reported previously (Dore, 2006) and the statistics of the diffraction data of crystals of condition B were better than that of condition A indicates the counter-diffusion gel capillary method produced better crystals than those grown in solution and by vapour diffusion. Our case study demonstrates that

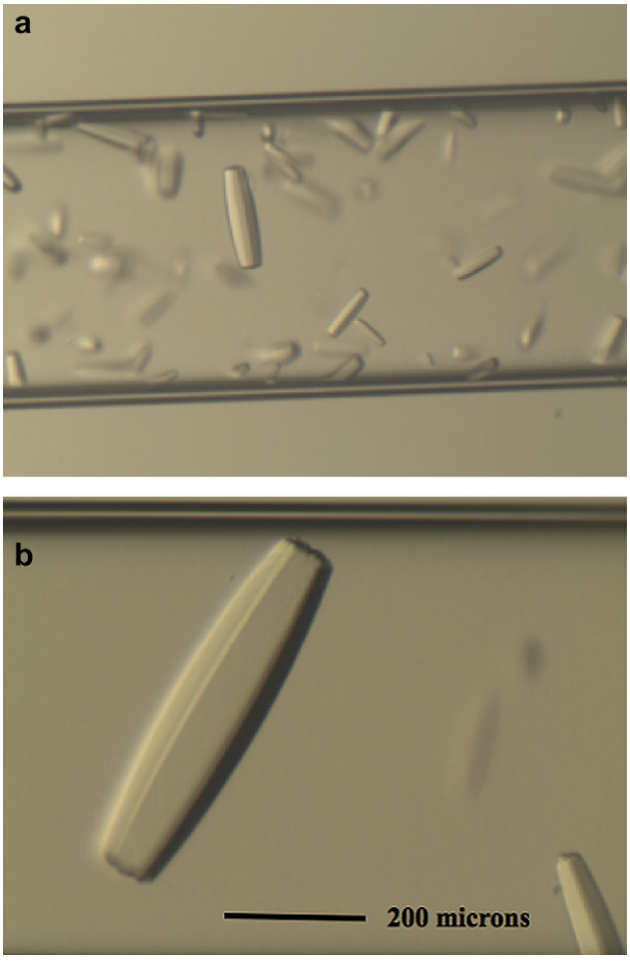

Fig. 5. (a) Crystals of Lif1p–Lig4p grown in agarose gel and capillary tubes. (b) Close up of one cryo-protected crystal. The diameter of the capillary tube in (a) is 0.5 mm.

Table 1
Diffraction data statistics of crystals obtained under conditions A and B.

	Condition A	Condition B
Beamline	ID13(ESRF)	ID14-4(ESRF)
Wavelength (Å)	0.968	0.979
Resolution (Å)	50–3.9	
Space group	$P6_422$	
Unit cell parameters (Å)		
a	247.62	246.86
b	247.62	246.86
c	98.42	98.78
Reflections (unique)	166,810 (16,475)	170,511 (16,699)
Completeness	99.8 (99.6)	99.3 (99.8)
Rmerge	10.1 (70.7)	5.3 (29.3)
$I/\sigma(I)$	5.7	13.5
$I/\sigma(I) > 3$	72.0 (36.2)	88.9 (69.5)
Mosaicity	0.210	0.194

the quality of protein crystals can improved by changing the crystallisation method without changing the composition of the crystallisation solution. One of merits of the counter-diffusion gel capillary method is that the original precipitant solution can be replaced by a similar solution containing ligands, dehydration solutions, cryoprotectant, etc. This in turn minimises crystal damage, as single crystals do not have to be manipulated manually.

4. X-ray topography as a tool for protein crystal quality X-ray characterisation

Commonly X-ray oscillation methods are used to characterise the crystal quality through the determination of the diffraction resolution, mosaicity and signal-to-noise. These values are often subjective or represent a convolution of several contributing factors. Further combination of the methods mentioned above is needed to better understand and characterise the macromolecular growth process, from early nucleation stages through growth cessation. Crystals that diffract to higher resolution, that is, for which smaller spacing in reciprocal space can be determined, with smaller mosaicities those with high signal-to-noise are considered to be of better quality then those which diffract to low resolutions, present high mosaicities and low signal-to-noise. So a crystal diffracting to 0.09 nm (0.9 Å) is considered of better quality than one diffracting to 0.3 nm (3 Å); or a crystal with 0.3° mosaicity as much worse than a crystal of 0.1° mosaicity. These dimensions, however, are average and only allow for qualitative information. In the search for quantitative information several groups have been pursuing X-ray diffraction imaging techniques (Bogon et al., 2000). Unfortunately, these methods are not readily accessible and require specialized experimental set-ups. A more accessible method developed by Lovelace and Borgstahl (2003) uses the oscillation method but instead of rotating the crystal in relatively large angular steps the sample is rotated in very small angular steps, typically of the order of millidegrees and the intensity for each reflection is integrated over this angular distance. It is then possible to build a distribution of intensities as a function of angular position, i.e., a rocking curve, for pre-determined reflections.

Simple optical observations of crystals often reveal lines marking the boundaries between different growth sectors (Robert and Lefaucheux, 1983) of biomacromolecular systems (Belouet et al., 1983; Robert et al., 2003; Chernov, 1984). The cause of such features has been investigated for the model material protein hen egg white lysozyme (HEWL), whose tetragonal crystal displays prismatic {110} and pyramidal {101} faces (Vekilov and Rosenberger, 1996) with the corresponding growth sectors. For crystals growing in impure solutions, the prismatic sectors appear striated by macroscopic growth bands and the pyramidal sectors are striation free, so that the boundaries between both sectors are clearly visible. As shown for small molecule crystals, impurities play an important role in the generation of growth bands: with purer solutions, the growth bands are less visible and the entire crystal seems more homogeneous. Further experimental studies have documented the role played by impurities on crystal growth, sometimes called "natural" impurities, which frequently appear in inorganic as well as protein molecules (Robert and Lefaucheux, 1988). Finally, the use of X-ray topographic analysis is one of the most precise methods for testing crystal quality. It is our hope that the methodologies proposed here will guide a protein crystallographer to find the appropriate strategy to obtain high-resolution crystals for biological investigations.

5. Molecular biology in protein crystallisation and concluding remarks

The fact that compounds that stabilise the structure or conformation of proteins such as cosolvents, ions, osmolytes, chaotropes and detergents (Trakhanov and Quiocho, 1995; Bolen, 2004, 2004; Arakawa and Timasheff, 1985; Schein, 1990; Zulauf et al., 1989) can promote crystal growth, has stimulated the search of a new generation of additives. When such additives are suitable for high-throughput platforms, they greatly extend the number of crystallisation conditions to screen, increasing the probability of identifying single crystals from the initial hits. Indeed, the use of small molecules to promote crystal growth is the basis of some commercial crystallisation kits such as the Silver Bullets Screen and the additives kit (Hampton Research, California, USA) (McPherson and Cudney, 2006). Furthermore, the compound I3C (5-amino-2,4,6-triiodoisophthalic acid) which is used for heavy-atom derivatization for SAD or SIRAS (single isomorphous replacement plus anomalous scattering) phasing rise as an interesting approach. This approach is refereed to as the magic triangle because it combines an arrangement of three anomalous scatterers (i.e., three covalently bound iodines) with functional groups for hydrogen bonding to a protein molecule. Chayen and collaborators have proposed a different approach, which relies in the identification of substrates that act as heterogeneous nucleant agents thus promoting the nucleation of protein crystals (Chayen et al., 2006). Initial efforts are currently underway to automate the delivery of nucleation-promoting substrates into the crystallisation droplet either in small-scale or in high-throughput platforms (Thakur et al., 2007; D'Arcy et al., 2007).

Other approaches widely used consist of the modification of the native protein using molecular biology methods and/or the incorporation of posttranslational modifications such as methylation or substitution of solvent exposed polar residues. The latter strategy, also referred to as rational protein surface engineering (Derewenda, 2004), seems particularly suitable for soluble proteins, were two or more mutations of the class K to A and/or E to A have been introduced. Mutation of these two polar amino acid residues is preferred because lysine and glutamate residues are found predominantly on the protein's surface, with only 6% and 12%, respectively, buried (Baud and Karlin, 1999). Based on the examples of successful applications, tight turns offer the best opportunities for crystal contact engineering (Baud and Karlin, 1999). However, as a cautionary tale it has to be said the K to A and E to A mutations almost invariably lower the protein's solubility and at least in the case of the E to A mutation, the substitution often results in a less stable protein (Mateja et al., 2002). Even though is advantageous that protein crystal quality is not necessarily correlated with intrinsic thermodynamic stability (Sippel et al., 2008), the systematic exploration of surface engineering on a wide range of proteins is

required to refine the strategy and make it more powerful and effective.

Finally, even in the most difficult cases, where the crystal growth method is not the total solution of the crystallisation problem, it might be coupled to molecular biology techniques to produce new constructs suitable for crystallisation. Sometimes, new crystalline polymorphs can be produced by mutating the native structure. Although we can conclude that there is no a single universal method for the crystallisation of biological macromolecules, this focused volume provides an encouragement to search for new possibilities to achieve a proper crystal quality in *de novo* proteins or macromolecular complexes, moving the reader from the classic methods to new strategies in capillary tubes, gels or controlling the nucleation or the kinetics of the process by means of physical parameters like electric or magnetic fields.

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