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Post-crystallization treatments for improving diffraction quality of protein crystals

Begoña Heras* and Jennifer L. Martin

Institute for Molecular Bioscience and ARC Special Research Centre for Functional and Applied Genomics, University of Queensland, Brisbane QLD 4072, Australia

Correspondence e-mail: b.heras@imb.uq.edu.au

Begoña Heras obtained her PhD degree from The University of Navarra (Spain), for her work on computer-assisted antidepressant drug design. She is currently a postdoctoral scientist at the Institute for Molecular Biosicence at the University of Queensland (Australia). Her research interests include protein structure and function particularly with regard to protein folding, protein–protein interactions and drug design. Jenny Martin is an Associate Professor at the Institute for Molecular Bioscience, University of Queensland. Her research interests include protein structure, folding and evolution and protein:ligand interactions. Her technological interests include high throughput crystallography and drug design. Jenny received a DPhil from the University of Oxford, UK.

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X-ray crystallography is the most powerful method for determining the three-dimensional structure of biological macromolecules. One of the major obstacles in the process is the production of high-quality crystals for structure determination. All too often, crystals are produced that are of poor quality and are unsuitable for diffraction studies. This review provides a compilation of post-crystallization methods that can convert poorly diffracting crystals into data-quality crystals. Protocols for annealing, dehydration, soaking and cross-linking are outlined and examples of some spectacular changes in crystal quality are provided. The protocols are easily incorporated into the structure-determination pipeline and a practical guide is provided that shows how and when to use the different post-crystallization treatments for improving crystal quality.

1. Introduction

Knowledge of the three-dimensional structure of proteins is essential in understanding their biological role and underpins downstream applications such as the design of new drugs. More than 85% of the macromolecular structures currently held in the Protein Data Bank have been determined by X-ray crystallography, making X-ray diffraction by far the most successful method for determining the structures of large molecules. A major stumbling block in this approach is the requirement for high-quality crystals. Despite technical and methodological advances in the field, including the development of highly focused X-rays, high-throughput crystallization techniques and automated X-ray data analysis, crystal growth still remains an empirical and tedious process and a common occurrence is the production of well formed crystals that are not suitable for diffraction studies. Loose packing of molecules and large solvent volume are common problems that result in low-resolution and poor-quality diffraction.

What are the options if crystals form but do not diffract well? Strategies that have been used in the past include searching for new crystallization conditions to identify a new crystal form, crystallizing the protein of interest from a different organism, crystallizing a different form of the protein by using proteases that produce smaller protein fragments, generating new constructs encoding a truncated form of the protein or mutating surface amino acids to enhance protein crystallization (Longenecker *et al.*, 2001). These methods all have one thing in common: they each give up on the crystals that have already been grown. However, before capitulating, there are several quick and simple methods that could be considered. Post-crystallization soaking, cross-linking, crystal annealing and controlled dehydration have been reported to

dramatically improve diffraction resolution of protein crystals. This review provides an overview of the different methods that can be used when faced with the all-too-frequent situation of protein crystals with poor-quality diffraction and provides a practical guide to implementing the procedures.

2. Crystal annealing

Protein crystals are sensitive to X-ray radiation and their diffraction quality rapidly deteriorates after being exposed to high-intensity X-rays. Data collection at cryogenic temperatures (around 100 K) has become a vital method for protein crystallography since it reduces radiation damage and increases crystal lifetime, allowing whole data sets to be collected from a single crystal (Hope, 1988, 1990; Rodgers, 1997; Garman, 1999). Protein crystals typically contain 50% water by mass and if cooled too slowly the water undergoes a large volume change associated with the transition from liquid water to hexagonal ice, resulting in degradation of crystal quality (Kriminski *et al.*, 2002). Ice formation and consequent crystal damage can be avoided by flash-cooling the crystal to produce an amorphous or vitrified form of water. Nevertheless, flash-cooling techniques can introduce lattice disorder, resulting in increased mosaicity and reduced diffraction resolution (Rodgers, 1994; Garman & Schneider, 1997). This damage, which is especially acute for large crystals and crystals with high solvent content, is thought to be a consequence of uneven cooling and differential expansion of the solvent and the crystal lattice (Juers & Matthews, 2001; Kriminski *et al.*, 2002).

Approaches for reducing mosaic spread caused by flash-cooling include a systematic exploration of cryoprotectants, sequential soaking of crystals in increasing concentrations of cryosolutions and control of the flash-cooling rate (Garman, 1999). However, a rapid and easy method to reduce flash-cooling-induced disorder that can increase diffraction quality of protein crystals has been described. This method, called crystal annealing, involves warming the flash-cooled crystal to room temperature and flash-cooling it again (Harp *et al.*, 1998). Three different crystal-annealing protocols have been reported. Macromolecular crystal annealing (MCA) consists of removing a cryocooled crystal from the cold gas stream and placing it in 300 µl cryosolution. After 3 min equilibration, the crystal is recooled in the cryostream (Fig. 1) (Harp *et al.*, 1998). The flash-annealing (FA) method involves blocking the cold-stream for 1.5–2 s three times with intervals of 6 s between each thawing step (Fig. 1) (Yeh & Hol, 1998). Annealing on the loop (AL), a variation of the flash-annealing method, also involves blocking the cryostream, but in this case the length of time varies from crystal to crystal: the cold-stream is blocked until the drop becomes clear, which is an indication that it has reached ambient temperature. When the cold nitrogen-gas stream is blocked, the flash-cooled drop becomes covered in ice, thus turning the drop opaque. As the drop warms to room temperature, the drop becomes clear (Harp *et al.*, 1999). AL does not use multiple cycles of warming and flash-cooling; a single annealing step will do (Fig. 1). Harp and coworkers used

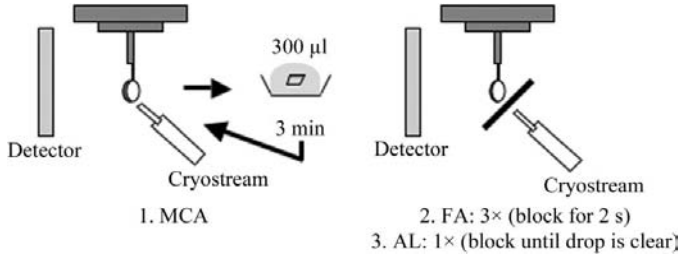

Figure 1
Annealing. Schematic outline of crystal-annealing procedures that can improve crystal quality. Macromolecular crystal annealing (MCA): remove cryocooled crystal from the cryostream, place it in 300 µl of cryosolution for 3 min and then recool (Harp *et al.*, 1998). Flash annealing (FA): block the cold-stream for 1.5–2 s three times with intervals of 6 s between thawing steps (Yeh & Hol, 1998). Annealing on the loop (AL): block cold-stream until the crystal becomes clear and then flash-cool again (Harp *et al.*, 1999).

crystals from six different proteins to evaluate the three annealing protocols and concluded that MCA treatment gives better and more reproducible results, FA was inadequate for most crystals and AL was successful with small crystals with low solvent content (Harp *et al.*, 1999). A more recent annealing protocol, also carried out in the loop, restricts the warming temperature to below the bulk-solvent melting point (230–250 K) and this was reported to give more reproducible improvement in the diffraction quality of protein crystals (Kriminski *et al.*, 2002). In this case, the temperature of the cryostream is quickly increased to 230–250 K and maintained until the crystal is equilibrated with the mix of cold and warm gas (∼10 s) prior to recooling. Application of this approach requires a system that permits regulation of the temperature in the cryostream.

A number of researchers have reported success in extending the diffraction resolution and reducing mosaicity of protein crystals by crystal annealing. Table 1 summarizes several examples, providing information about the specific methods used as well as the improvements achieved. In some cases, this post-crystallization treatment has produced spectacular results. For example, in the case of three different proteins, *N*-acetylglucosamine 6-phosphate deacetylase (GlcNAc6P), dmpFG-encoded 4-hydroxy-2-ketovalerate aldolase-aldehyde dehydrogenase and arsenate reductase, the diffraction resolution improved from ∼6 Å (medium resolution in the case of GlcNAc6P) to ∼2 Å upon crystal annealing (Table 1). Furthermore, for copper nitrite reductase and F1-ATPase the annealing procedure reduced the mosaicity from more than 1° to ∼0.3° (Table 1).

3. Crystal dehydration

Water plays a crucial role in maintaining the structure and activity of protein molecules both in solution and in crystalline form (Frey, 1994; Timasheff, 1995). Crystallographers have investigated in detail the water-mediated transformations in protein crystals and it is well known that reduction of solvent content can produce more closely packed and better ordered

Table 1
Summary of the effect of crystal annealing on different protein crystals.

EG, ethylene glycol; MME, monomethyl ether; MPD, 2-methyl-2,4-pentanediol; NR, not reported; Paratone, Paratone-8277 (previously known as Paratone-N); PEG, polyethylene glycol.

Protein crystal†	Space group	Precipitant‡	Cryoprotectant	Solvent content (%)	Resolution before/after (Å)	Mosaicity before/after (°)	Annealing method§	Reference¶
Nucleosome core particle	$P2_12_12_1$	$MnCl_2$	22.5% MPD/Paratone	51	NR/3.1	0.82/0.34	MCA	*a*
Histone octamer	$P3_221$	70% $(NH_4)_2SO_4$	15% glycerol/Paratone	65	NR/3.0	0.34/0.22	MCA	*a*
Glycerol kinase	$C2$	35% PEG 4K/7.5% PEG 200	20% EG	NR	3.7/2.8	>2/∼1	FA	*b*
Inorganic pyrophosphatase	$R32$	1.4–2 *M* NaCl	27–30% glycerol	∼40	1.8/1.15	0.70/0.30	MCA [salt]	*c*
GlcNAc6P	$P2_12_12$	1.85 *M* NaH_2PO_3	20% glycerol	46	Medium/2.0	NR	MCA	*d*
DmpFG	$P2_12_12_1$	15% PEG 8K	Paratone	48	>6/2.1	NR	MCA	*e*
α-Glucosidase	$P2_1$	20–25% PEG 4K	20% PEG 4K/5% glycerol	35	∼3/2	NR	FA	*f*
Arsenate reductase	$P2_12_12_1$	30–35% PEG MME 2K	30–35% PEG MME 2K	47	6.0/2.3	NR	FA	*g*
dUTP pyrophosphatase	$P2_12_12_1$	23.4% PEG 1.5K	40% PEG 1.5K	63	∼3/2.2	NR	MCA	*h*
Copper nitrite reductase	$P6_3$	40–50% PEG MME 550	40–50% PEG MME 550	NR	2.5/1.0	1.5/0.3	AL	*i*
Lipoprotein receptor LolB	$P2_1$	30% PEG MME 2K	30% PEG MME 2K	44	NR/1.9	NR	FA	*j*
TAXI I endoxylanase inhibitor	$P2_12_12_1$	23% PEG 4K	20% glycerol	47	NR/1.75	NR	AL	*k*
F1-ATPase	$P2_1$	11% PEG 6K	Paratone	55	NR/2.8	∼1/0.28	AL	*l*

† GlcNAc6P, *N*-acetylglucosamine 6-phosphate deacetylase; DmpFG, dmpFG-encoded 4-hydroxy-2-ketovalerate aldolase-aldehyde dehydrogenase; dUTP pyrophosphatase, deoxyuridine triphosphate nucleotidohydrolase. ‡ Does not include information about buffers or additives. § MCA, macromolecular crystal annealing; FA, flash-annealing; AL, annealing on the loop. ¶ *a*, Harp *et al.* (1998); *b*, Yeh & Hol (1998); *c*, Samygina *et al.* (2000); *d*, Ferreira *et al.* (2000); *e*, Manjasetty *et al.* (2001); *f*, Vévodová *et al.* (2001); *g*, Guan *et al.* (2001); *h*, Han *et al.* (2001); *i*, Ellis *et al.* (2002); *j*, Takeda *et al.* (2003); *k*, Sansen *et al.* (2003); *l*, Mueller *et al.* (2004).

crystals, extending the resolution of X-ray diffraction patterns (Salunke *et al.*, 1985; Frey, 1994). Indeed, after reviewing different post-crystallization methods to improve crystal diffraction, crystal dehydration emerges as the treatment that has produced the most remarkable improvements in the diffraction resolution of protein crystals (Heras *et al.*, 2003; Abergel, 2004). Some of these examples are summarized in Table 2. It should be noted that while our literature search yielded these 18 examples, this is by no means a comprehensive list and there may be other cases that we have missed.

Several different protocols have been developed for crystal dehydration. The first dehydration experiments were carried out by Perutz, who allowed crystals mounted in a capillary tube to lose water to the atmosphere through a small hole made in the tube (Perutz, 1946; Bragg & Perutz, 1952). This method was further improved by connecting the glass capillary to a salt reservoir so that by changing the salt concentration the relative humidity in the capillary could be varied and different crystal forms produced (Huxley & Kendrew, 1953). In that paper, Huxley and Kendrew also described an apparatus for controlled shrinkage of protein crystals. In another example, Madhusudan and coworkers reduced the solvent content in monoclinic lysozyme from 33 to 22% by placing a few drops of K_2CrO_4 solution in the glass capillary; this improved the diffraction resolution from 2.5 to 1.75 Å (Madhusudan *et al.*, 1993). Similarly, the response of tetragonal lysozyme to dehydration was characterized by mounting crystals in X-ray capillaries and equilibrating them against different saturated salt solutions (various salts were used to achieve relative humidities ranging from 97 to 75%; Dobrianov *et al.*, 2001).

Crystal dehydration can also be performed by transferring the crystals into a dehydrating solution, which is usually the original mother liquor either with a higher concentration of precipitant or supplemented with cryoprotective agents such as PEG 400, PEG 600, glycerol or MPD. It should be noted that one mechanism of cryoprotection is to reduce protein solvation, so that cryoprotectants can act as dehydratants and *vice versa*. On the other hand, cryoprotectants such as glycerol can also act as co-solvents, increasing protein solubility. Further information about cryoprotectants and their mechanisms can be found in Timasheff & Arakawa (1988), Charron *et al.* (2002) and Mi *et al.* (2004).

The first example of crystal dehydration performed by moving the crystal into dehydrating solution was reported by Schick & Jurnak (1994). They improved the diffraction resolution of the guanidine nucleotide-exchange factor complex from 4 to 2.5 Å (Table 2) by a serial transfer of the protein crystal to droplets (50 µl) of cryoprotective agent of increasing concentration, with incubations of 5 min in each condition (method 1; Fig. 2). In contrast, Haebel *et al.* (2001) extended the diffraction resolution of crystals of a DsbC–DsbDα complex from 7 to 2.6 Å resolution (2.3 Å resolution at a synchrotron; Table 2) by the slow addition to the crystal droplet of a total of eight times the crystallization drop volume of dehydrating solution followed by equilibration against air for a period of 30 min (method 2; Fig. 2). Alternatively, the crystal can be transferred from the crystallization drop into a 5 µl hanging drop of dehydrating solution, which is then equilibrated for 12–16 h against the same dehydrating solution at 277 K (method 3; Fig. 2; Heras *et al.*, 2003). Crystals of DsbG dehydrated in this way exhibited a dramatic improvement in diffraction quality, with the pattern improving from 10 Å to beyond 2 Å resolution (Table 2). A more gentle dehydrating method consists of the serial transfer of the cover slip holding the crystal droplet over reservoirs containing increasing concentrations of dehydrating solution with incubations of 8–12 h over each condition (method 4; Fig. 2).

Table 2
Summary of the effect of crystal dehydration on different protein crystals.

AS, ammonium sulfate, cryst. drop, crystallization drop; EG, ethylene glycol; exp., exposure; HA, heavy atom; incub., incubation; incr., increment; MPD, 2-methyl-2,4-pentanediol; PEG MME, PEG monomethylether; NR, not reported; PEG, polyethylene glycol; ppt, precipitant; satd, saturated; sol., solution.

Protein crystal†	Space group	Precipitant‡	Dehydrating agent	Treatment (incubation time)	Solvent content before/after (%)	Resolution before/after (Å)	Reference§
EF-Tu-Ts	$P2_12_12_1$	20% PEG 4K	28–40% various PEGs	Method 1 (∼5 min)	61/55	4.0¶/2.7¶	*a*
NF-κB P52–DNA	$I2_12_12_1$	4–6% PEG 4K	Ppt+ 30% PEG 400 (+ HA)	Method 1	52/49	3.5††/2.0††	*b*
HIV(RT)–inhib.	$P2_12_12_1$	6% PEG 3.4K	46% PEG 3.4K	Method 1 (5% incr.; 3 d)	56/48	3.7¶/2.2¶	*c*
DsbC–DsbDα	$P4_32_12$	25% PEG MME 5K/ 5% glycerol	40% PEG MME 5K/ 10% glycerol	Method 2 (30 min)	55/41	7.0¶/2.6¶ (2.3††)	*d*
DsbG	$C2$	20% PEG 4K	30% PEG 4K	Method 3 (12 h)	∼90/53	10¶/2.0¶ (1.7††)	*e*
E. coli YbgL	$C2$	0.8 *M* sodium citrate	Ppt + 10% EG	Annealing/air dehydrate (2 h)	NR/57	12¶/2.6¶ (1.8††)	*f*
E. coli YggV	$P4_32_12$	35% $(NH_4)_2SO_4$	37.5% AS/10% glycerol	Annealing/air dehydrate (30 min)	NR/38	12¶/2.6¶ (2††)	*f*
3-Dehydro dehy	$P2$	11% PEG 8K	Ppt + 10% glycerol	Annealing/air dehydrate (15 min)	NR/88	None/3	*f*
Rv2002 product	$P3_121$	20% PEG 3K	Ppt + 10% MPD	Annealing/air dehydrate (5 h)	NR/35	2.1††/1.8††	*g*
Peptide deform	$P2_12_12_1$	12% PEG 4K	20% PEG 4K/ 10% PEG 400	Annealing/air dehydrate (30 min)	NR/50	2.0††/1.85††	*h*
HCMV prot	$P4_12_12$	16% PEG 4K	30% PEG 4K/Na_2SO_4	Method 1 (3–5 d)	58/56	3.0¶/2.5¶ (2.0††)	*i*
PDH	$R32$	6% PEG 3K	Ppt	Dehydrate/rehydrate	NR/73	7.0††/4.2††	*j*
FAD-indep ALS	$C2$	6–8% PEG 8K/ 6–9% EG	Ppt/30% PEG 600	Method 3/cryocool (24 h)	NR/52	2.9¶/2.6¶	*k*
Lysozyme	$P2_1$	3% $NaNO_3$	Satd K_2CrO_4 sol.	Method 4 (∼20 h)	33/22	2.5¶/1.75¶	*l*
Lysozyme	$P4_32_12$	0.48–0.75 *M* NaCl	Satd salt sol.	Method 4 (days/weeks)	NR	1.6††/3.7††	*m*
RFC–PCNA	$P2_12_12_1$	15% PEG 3350	33% PEG 3350	Method 4 (2 h)	58/52	5.0††/2.85††	*n*
CLC Cl channel	$P222$	22–32% Jeffamine	Ppt	Incub. in cryst. drop (5 months)	NR	7.5††/4.0††	*o*
Cytochrome ba_3 oxidase	$P4_32_12$	14–16% PEG 2K	20% glycerol/20% EG	Incub. under oil 2–4 h/ air exp. 10 min	NR/61.7	4.0††/2.3††	*p*

† EF-Tu-Ts, guanidine nucleotide-exchange factor complex EF-Tu-Ts; NF-κB P52–DNA, transcription factor NF-κB P52–DNA complex; HIV(RT)–inhib., HIV1 reverse transcriptase–inhibitor complex; 3-Dehydro dehy, *C. albicans* 3-dehydroquinate dehydratase; Peptide deform, peptide deformylase; HCMV prot, human cytomegalovirus protease; PDH, pyruvate dehydrogenase; FAD-indep ALS, FAD-independent acetolactate synthase; Lysozyme, hen egg-white lysozyme; RFC–PCNA, replicator factor C–proliferating nuclear antigen complex. ‡ Does not include information about buffers or additives. § *a*, Schick & Jurnak (1994); *b*, Cramer & Muller (1997); *c*, Esnouf *et al.* (1998); *d*, Haebel *et al.* (2001); *e*, Heras *et al.* (2003); *f*, Abergel (2004); *g*, Yang *et al.* (2002); *h*, Kim *et al.* (2002); *i*, Tong *et al.* (1997); *j*, Izard *et al.* (1997); *k*, Pang *et al.* (2002); *l*, Madhusudan *et al.* (1993); *m*, Dobrianov *et al.* (2001); *n*, Bowman *et al.* (2004); *o*, Kuo *et al.* (2003); *p*, Hunsicker-Wang *et al.* (2005). ¶ X-ray diffraction resolution on a rotating-anode source. †† X-ray diffraction resolution at a synchrotron source.

Combining dehydration with other post-crystallization treatments such as annealing, soaking, cryocooling or rehydration has also resulted in spectacular improvements in the diffraction quality and resolution of protein crystals (Table 2) (Izard *et al.*, 1997; Tong *et al.*, 1997; Pang *et al.*, 2002; Abergel, 2004). For example, a recent publication reported the dramatic improvement in diffraction resolution of three protein crystals upon annealing and dehydration [*Escherichia coli* YbgL from 12 to 2.6 Å, *E. coli* YggV (HAM1) from 12 to 2.6 Å and *Candida albicans* 3-dehydroquinate dehydratase from no diffraction to 3 Å]. The method involved removing the poorly diffracting crystal from the cryostream and placing it in a 10 µl droplet containing dehydrating solution (90% mother liquor and 10% cryoprotectant such as glycerol or ethylene glycol) followed by air drying from 15 min to 2 h (Table 2; Abergel, 2004).

Dehydration has also proven successful in improving the diffraction resolution of membrane-protein crystals. The diffraction limit and quality of prokaryotic CLC chloride channel crystals improved (resolution from 8 to 4 Å; mosaicity from >5 to 1°) upon slow dehydration over a period of months (Kuo *et al.*, 2003).

Finally, in addition to these dehydration methods, a device that controls the humidity surrounding the crystal has also been described and successfully utilized to improve diffraction of protein crystals (Kiefersauer *et al.*, 2000).

4. Other methods

4.1. Post-crystallization soaking without dehydration

Post-crystallization soaking is similar to crystal dehydration in that both processes involve soaking protein crystals in solutions containing increased precipitant concentrations or cryoprotectants. However, dehydration implies shrinking of the crystal lattice and lowering of the solvent content of the crystal, whereas post-crystallization soaking without dehydration does not involve a change in unit-cell or solvent content, yet still leads to a notable improvement of the diffraction quality of the crystal.

Several examples have been described where soaking crystals in higher ionic strength solutions, cryoprotectants or heavy-atom-containing solutions results in improvement in the quality of the crystals. For example, Fu and coworkers transferred MTCP-1 protein crystals grown in ammonium sulfate to fresh drops containing a higher salt concentration and incubated the crystals for one to five months. This extended the diffraction resolution from 3 to 2 Å (Fu *et al.*, 1998, 1999). They postulated that the improvement in

diffraction quality was a consequence of the rearrangement of surface residues to form better packing interactions. The method was further optimized by Petock and coworkers, who varied the composition of the soaking solution, combining ammonium sulfate with PEG 3400, which reduced the incubation time of MTCP-1 protein crystals to 1–10 weeks (Petock *et al.*, 2001).

Post-crystallization soaking in solutions containing cryoprotecting agents such as glycerol can also improve the quality of protein crystals (Sousa, 1995). Thus, Rould and coworkers reported that the diffraction limit of glutaminyl-tRNA synthetase-tRNA co-crystals could be increased by soaking in 20% glycerol followed by cooling to 265 K (Rould *et al.*, 1991). This treatment also improved the order in disordered regions of the crystal. Moreover, in a recent study on the versatility of malonate as cryoprotectant, it was found that soaking in 50% sodium malonate solution can eliminate crystal disorder and improve the resolution limit (Holyoak *et al.*, 2003).

Preparation of heavy-atom derivatives for phasing purposes (reviewed in Garman & Murray, 2003) can involve the immersion of protein crystals into heavy-atom-containing solutions. Heavy-atom derivatives produced in this way usually diffract less well than native crystals, although in some cases the resolution limit has been shown to improve. For example, Cramer and Muller reported that the anisotropic diffraction exhibited by NF-κB P52–DNA cocrystals could be corrected by soaking heavy-metal ions into the crystal (Table 2; Cramer & Muller, 1997). This soaking process was accompanied by unit-cell shrinkage, suggesting that dehydration could also have contributed to the change. However, improvements in diffraction resolution without apparent dehydration have also been reported upon heavy-atom derivatization. For example, the diffraction resolution of MscL protein crystals, a mechanosensitive ion channel from *Mycobacterium tuberculosis*, improved from 7 to 3.5 Å resolution upon soaking in heavy-atom compounds (Chang *et al.*, 1998).

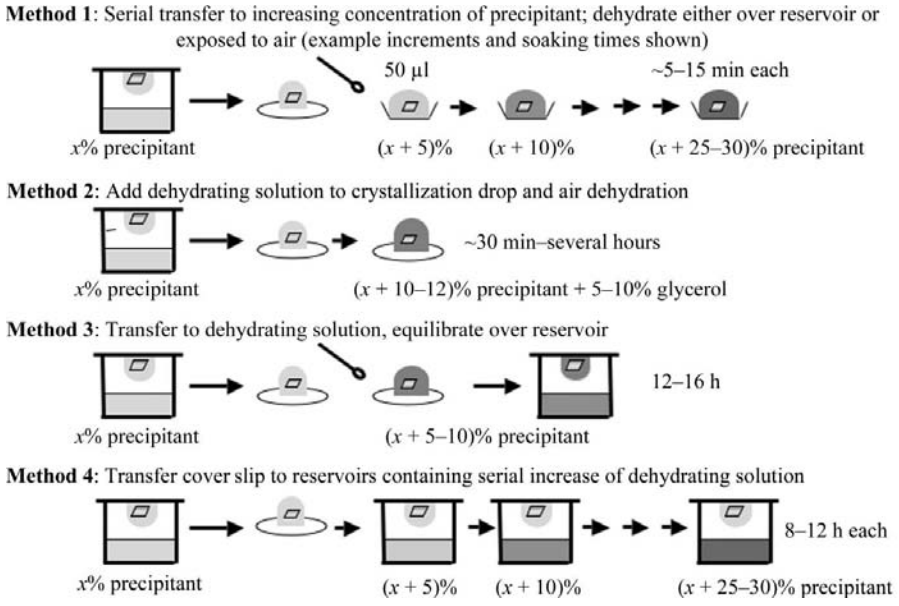

Figure 2
Dehydration methods. Method 1: serial transfer of crystals from the crystallization drop to 50 µl drops containing increasing amounts of dehydrating solution. The dehydrating solution can consist of mother liquor with increasing precipitant concentration or it can be supplemented with increasing concentrations of cryoprotective agents such as PEG 400, glycerol or MPD. Depending on the stability of the crystal, the concentration of dehydrating agent can be increased in steps of 5% up to ~30%(*w*/*v*), steps of 0.5% up to~5%(*w*/*v*) or as follows: 1, 2, 3, 4, 5, 10, 15, 20%. Soaking time can also vary from 5 to 15 min (in this case crystals are air dehydrated) to days (in this case dehydrating drops are equilibrated against a reservoir containing dehydrating solution) (Schick & Jurnak, 1994; Esnouf *et al.*, 1998). Method 2: add dehydrating solution slowly to the drop containing the crystal (about eight times the crystallization drop volume) and air dehydrate for more than 30 min. Dehydrating solution consists of crystallizing conditions with a 10–12% increase in precipitant concentration (*e.g.* PEG, MPEG) and ~5–10% addition of cryoprotective agent such as glycerol (Haebel *et al.*, 2001). Method 3: transfer the crystal from the crystallization drop into a 5 µl hanging drop of dehydrating solution and equilibrate against a reservoir with the same dehydrating solution (dehydrating solution: crystallization condition containing 5–10% more precipitating agent; incubation time: 12–16 h; Heras *et al.*, 2003). Method 4: After crystal growth, equilibrate the crystallization drop against reservoirs containing increasing concentration of dehydrating agent (dehydrating solution: mother liquor containing increasing concentrations of either precipitant or low-molecular-weight PEG, glycerol or MPD. Concentration is increased in steps of 5%. Incubation time: 8–12 h each). For very fragile crystals it is recommended that these dehydration procedures be performed at 277 K. Crystal soaking without dehydration is performed similarly to method 1 by, for example, transferring the crystal to 10 µl of soaking solution consisting of mother liquor containing a cryoprotectant (~20% glycerol or 40–50% malonate) and incubating for a few seconds to 5 min (Holyoak *et al.*, 2003).

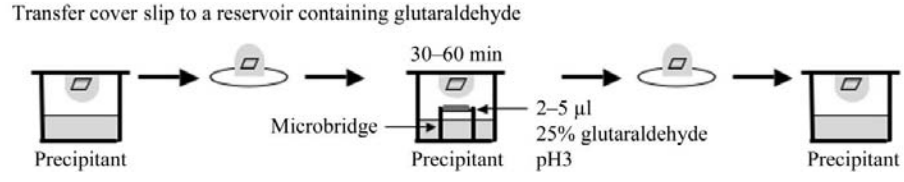

Figure 3
Crystal cross-linking by vapour diffusion. Transfer cover slip containing the crystal to a new reservoir with precipitating solution and a microbridge (Hampton Research) containing a sitting drop (2–5 µl) of 25% glutaraldehyde pH 3. Equilibrate for 30–60 min and stop the process by placing the cover slip over a new reservoir with fresh precipitant solution (Lusty, 1999).

4.2. Cross-linking

Data collection at cryogenic temperatures requires the addition of a

suitable cryoprotectant to the protein crystal. While some crystals can be directly dipped in cryoprotective solutions, other crystals do not tolerate this procedure. In this situation, it may be possible to chemically cross-link the protein crystal using glutaraldehyde or another cross-linking reagent before proceeding with the cryoprotection procedure (Quiocho & Richards, 1964). Crystal cross-linking increases the robustness of the crystal against mechanical stress, reduces its solubility (Quiocho & Richards, 1964) and can also improve diffraction quality.

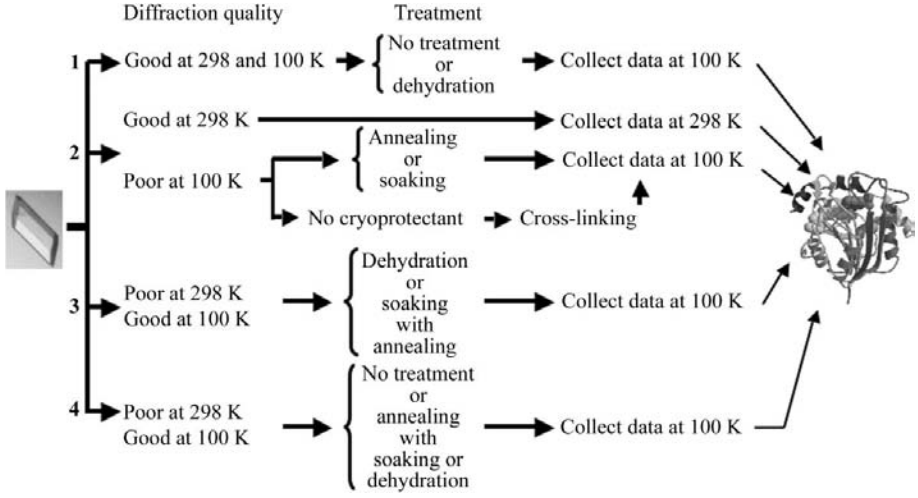

Figure 4
Flow diagram depicting practical experiments to perform on crystals with various diffraction qualities.

Cross-linking involves the reaction of lysine amines with aldehydes of the cross-linking (usually glutaraldehyde) molecule; improvement in diffraction quality depends on the position of the lysines in the crystal and the total number of lysines in the asymmetric unit (Lusty, 1999). The cross-linking reaction is pH-dependent: the more basic the solution the more susceptible the aldehyde is to condensation, which reduces the number of free aldehyde groups (Monsan *et al.*, 1975). Reproducibility and loss of diffraction owing to the use of excessive cross-linking agent are also common problems with this post-crystallization treatment. A more gentle cross-linking method has been described in which glutaraldehyde is introduced into the crystal by vapour diffusion (Lusty, 1999). The process is carried out by equilibrating a hanging droplet holding the crystal over a reservoir containing precipitant solution and a microbridge (Hampton Research) holding 2–5 µl of 25% glutaraldehyde (Fig. 3). The process was evaluated on crystals of three different proteins. This showed not only that the process was highly reproducible, but also that crystal cross-linking can prevent lattice disorder caused by cryocooling. Crystal diffraction of a selenomethionyl *N*-cadherin fragment (2.9 Å) and a DNA complex of MMLV reverse transcriptase (1.9 Å) was similar before and after cross-linking, but for a HIV-1 gp120 ternary complex diffraction improved from 2.7 to 2.2 Å. Strikingly, mosaicity was substantially reduced in all three cases after cross-linking (from 2–5° to 0.4–1°; Lusty, 1999). A similar cross-linking approach was used to improve the diffraction properties of protein–DNA complex crystals (Reményi *et al.*, 2001). In this case the resolution improved from 3.2 to 1.9 Å and the mosaicity decreased from ∼2 to 0.5° after glutaraldehyde cross-linking.

Another cross-linking method that has been described involves equilibrating the protein crystal against a reservoir containing mother liquor supplemented with 0.125% glutaraldehyde (2–3 h; Jacobson *et al.*, 1996).

5. Practical suggestions

How can we best make use of these methods to improve crystal quality? The purpose of this section is to provide a protocol for selecting the most appropriate post-crystallization treatment. We have classified protein crystals into four different categories according to their diffraction quality at cryo- and room temperature and for each category suggested the most suitable treatment (Fig. 4).

5.1. Category 1

This is the ideal case; crystals have high-quality diffraction patterns at both room and cryo-temperatures (Fig. 4). In this situation no post-crystallization treatment is necessary, although crystal dehydration might be worthwhile as a quick option to further extend crystal diffraction. The choice of dehydrating method will depend on the robustness of the crystal. For delicate crystals, more gentle methods are recommended such as methods 3 or 4 (Fig. 2). For robust crystals, quicker dehydration methods can be attempted such as methods 1 or 2 (Fig. 2). In all these methods the dehydrating agent used is the crystallization condition with a higher precipitant concentration or with added cryoprotective agents (such as low-molecular-weight PEGs, glycerol or MPD). In Fig. 2, specific concentrations of dehydrating agent are suggested for each dehydrating method. These values are based on published data as well as our own experience; however, these concentrations should be used as a guide only and may be adapted to the specific requirements of each crystal.

5.2. Category 2

In this case, crystals diffract to high resolution at room temperature but poorly at 100 K (Fig. 4). If the cooling process significantly affects the diffraction quality of the crystal, data collection at room temperature could be an option, although radiation damage to the protein crystal may limit the diffraction resolution and the quality of the final three-dimensional structure. If cryo data measurement is preferred, the post-crystallization treatment to try first is crystal annealing (Fig. 1). Annealing on the loop is the quickest

method since it simply involves blocking the cryostream until the drop becomes clear and then flash-cooling the crystal again. Macromolecular crystal annealing is also recommended since this method has yielded the greatest improvements in diffraction quality of protein crystals upon annealing. If crystal annealing does not yield improved results, serial transfer of the crystal into increasing concentrations of cryoprotectant can be attempted. This treatment reduces the osmotic shock suffered by the crystal when exposed to the cryoprotecting buffer and may improve crystal diffraction at cryo-temperatures. Also, different types of cryoprotectants should be tried (Garman & Schneider, 1997; Garman, 1999). Oils such as Paratone-N can also be utilized as a cryoprotectant for biological macromolecular crystals (Hope, 1988) and these also can have a dehydrating effect on protein crystals, which may improve their diffraction resolution. If no suitable cryoprotectant can be found, then cross-linking of protein crystals prior to cryoprotection is recommended (Fig. 3).

5.3. Category 3

This category includes those crystals that diffract poorly at both 298 and 100 K. Here, it is worthwhile investigating different post-crystallization treatments. Crystal dehydration should be attempted first since, to the best of our knowledge, this is the post-crystallization treatment that has resulted in the most dramatic improvements in diffraction resolution of protein crystals (Table 2, Fig. 2). Once again, the selection of the dehydration method will depend on the stability of the crystal. For salt-grown crystals, soaking in higher salt concentrations supplemented with 5–10% PEG 3350 should be tried. Crystal soaking in malonate is also a good option since this may not only improve the diffraction resolution, but this particular salt can also act as a cryoprotectant. For crystals in category 3, annealing only is unlikely to improve the diffraction quality since their poor resolution is not entirely a consequence of the cooling process. However, combinations of one of the previous treatments (dehydration and soaking) with crystal annealing are recommended (Fig. 4).

5.4. Category 4

In the unlikely case that crystals diffract well only at cryo-temperature but not at room temperature, no post-crystallization treatment is required. However, we suggest investigating crystal annealing since the thawing and recooling process may further increase resolution. Also, a combination of annealing with either crystal soaking in cryoprotectant or crystal dehydration might be worth trying to further extend the diffraction resolution.

6. Summary

On a final note, given the spectacular results obtained by crystal dehydration and the simplicity of this particular approach, we recommend using this procedure routinely as a method to potentially improve the diffraction limit of protein crystals. Crystals can be dehydrated in many different ways and the selection of the method will depend on the stability of the crystal. For robust crystals, methods 1 and 2 are recommended, whereas methods 3 and 4 are more suitable for delicate crystals. It should also be noted that methods 2 and 4 could easily be incorporated into a high-throughput structure pipeline.

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