Quarterly Reviews of Biophysics **37**, 2 (2004), pp. 1–15. © 2004 Cambridge University Press
DOI: 10.1017/S0033583504004007 Printed in the United Kingdom

ESSAY

Cryo-cooling in macromolecular crystallography: advantages, disadvantages and optimization

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Abstract. The flash-cooling of crystals in macromolecular crystallography has become commonplace. The procedure makes it possible to collect data from much smaller specimens than was the case in the past. Also, flash-cooled crystals are much less prone to radiation damage than their room-temperature counterparts, allowing data to be accumulated over extended periods of time. Notwithstanding the attractiveness of the technique, it does have potential disadvantages. First, better methods need to be developed to prevent damage to crystals on freezing. There is also a risk that structures determined at low temperature may suggest conclusions based on aspects of the structure that are not necessarily relevant at room temperature.

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1. The motivation for cryo-cooling

The determination of macromolecular structures via X-ray crystallography has grown tremendously in the last decade. The structures of increasingly complicated macromolecules are being reported, many thought nearly impossible to determine only a few years ago. Methods for protein expression and purification, crystal growth, cryo-cooling, data collection, data processing, phase determination and structure refinement have all seen improvements, in many cases to the point of automation. This growth resulted from a combination of empirical observation, improved theoretical understanding and clever experimentation, driven by the ever-present desire to speed-up the process of structure determination.

A major shift making many of these structure determinations possible is the now nearly universal practice of cooling crystals to cryogenic temperatures for the purpose of reducing the rate of radiation damage during X-ray data collection. Using lower temperatures to reduce radiation damage has been known for a long time (Low *et al.* 1966), but has become the method of choice only in the last few years. With the increasing availability of high-intensity synchrotron radiation sources and experiments requiring large X-ray doses, such as multiple-wavelength phasing and the use of smaller crystals, cooling is usually required to record quality data at high resolution.

Cooling crystals to low temperatures has two consequences we would like to consider here.

1.1 Damage introduced by cooling

Although cooling can slow lattice degradation due to the ionizing radiation, it is itself often damaging to the crystal. Sometimes the damage from cooling can be kept to a minimum, or even reversed, by altering the cooling procedure. In other cases, however, adequate cooling conditions cannot be found, precluding structure determination. An important challenge for the field of macromolecular crystallography is to understand the nature of this cooling-induced damage and to use this knowledge to successfully cool crystals of interest. Recent experiments have started to shed some light on cooling-induced damage and its recovery, and further study should help to improve cooling procedures.

1.2 Biological relevance of low-temperature structures

The large majority of macromolecular structures are now determined at temperatures $\sim$200 K below that of their *in vivo* milieu (Juers & Matthews, 2001; Halle, 2004). Thus, some consideration needs to be given as to how relevant structures determined at 100 K are for answering questions about biology at 300 K.

2. Basics of cryo-cooling

Several different methods for cooling macromolecular crystals have been developed. For an overview we refer the reader to the review by Garman & Schneider (1997). Currently the most common method is to mount the crystal with a polyethylene loop via surface tension and cool the crystal either by submerging it in a liquid cryogen or placing it directly into a cold gas stream (Teng, 1990). This usually vitrifies the bulk solvent, slowing diffusion of damaging free radicals produced by the ionizing radiation. The popularity of this method derives from its ease of use and reasonable success rate. Within this method, however, there is the possibility of considerable variation, including the choice of temperature, the cooling rate, the identity and phase (liquid

or gas) of the cryogen, the composition and amount of solution surrounding the crystal, and the crystal size. There are even more variables when the possibility of recovering from cooling-induced damage by cycling the crystal between low and high temperature is considered.

Usually the crystal is prepared for the cooling process with one or both of two methods. In the first, the solution surrounding the crystal is removed by passing the crystal through some kind of hydrocarbon-based liquid (Hope, 1988). This procedure can be facilitated by using a finely drawn glass tube to remove mother liquor from the outside of the crystal, leaving, in principle, bulk solvent only in the solvent channels, and the surface of the crystal bathed in the hydrocarbon. This method can eliminate the formation of ice in the surface mother liquor. In the second method, the crystal is either equilibrated to, or grown out of, a solution containing cryoprotectant. Cryoprotectants can prevent ice formation and alter the bulk thermal contraction properties of the solvent bathing the crystal. The possibility of using both oils and cryoprotectants together has also been suggested and has shown some promise, but needs to be investigated more thoroughly (Kwong & Liu, 1999; Juers & Matthews, 2004).

Other methods for cooling have been described, which although not very commonly used, are interesting from a physical point of view. These include cooling very slowly, using solvents which remain liquid at the low temperature to allow for diffusion of ligands, and cooling under high pressure to prevent solvent expansion (Petsko, 1975; Walter *et al.* 1982; Parak *et al.* 1987).

3. Consequences of cryo-cooling

3.1 Changes in the diffraction pattern

The successful addition of cryosolvent should cause little discernible change in the room-temperature diffraction pattern, other than a possible change in spot separation, reflecting a change in cell dimensions. However, there are usually changes in the diffraction pattern after cooling. Spot widths may broaden, often anisotropically, and peak intensities may decrease. The amount of diffuse scatter often increases. The diffraction limit for a single image may decrease, although higher resolution datasets can still be collected due to the decreased radiation damage rate. Below we discuss some of the changes in the diffraction pattern in the context of the macromolecule, the lattice and the solvent.

3.2 Changes in the macromolecule and macromolecular lattice

When a macromolecular crystal is cooled the constituents can respond in different ways. The macromolecules themselves contract (Frauenfelder *et al.* 1987) and a survey of crystal structures suggested that on average, proteins contract by 1–2% in volume when cooled to $\sim$100 K (Juers & Matthews, 2001). The unit cell also contracts, but by a greater amount – on average $\sim$4–5% in volume. Additionally, the crystal lattice usually repacks (Fig. 1). The amount of repacking varies widely, ranging from almost no changes in intermolecular contacts to large-scale remodeling of the contacts that can increase the surface buried at the contacts by 50% (Juers & Matthews, 2001). Occasionally, the lattice repacks in such a way that the space group changes between room- and low-temperature (Campobasso *et al.* 1998).

At length-scales larger than a single unit cell, macromolecular crystals are often thought of as assemblages of domains (Darwin, 1922; Nave, 1998; Boggon *et al.* 2000). The domains are composed of a finite number of unit cells. Each domain is assumed to be a perfect crystal, but

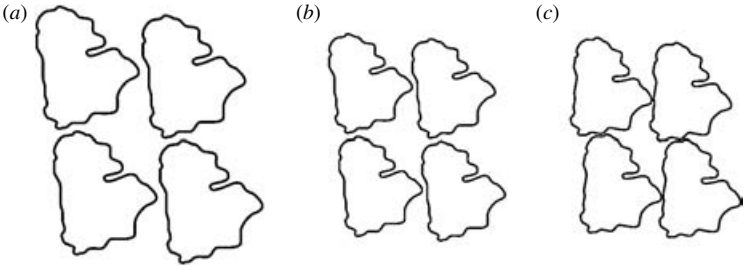

Fig. 1. Schematic showing shrinkage of the macromolecule and the lattice. (*a*) The crystal lattice at room temperature. (*b*) With cooling, the individual macromolecules contract ∼1–2%. Here, a hypothetical low-temperature crystal is shown in which the room-temperature packing is maintained, with lattice contraction of 1–2%. (*c*) Usually, the lattice repacks with cooling, further shrinking the unit-cell volume by 4–5% from its original room-temperature value.

domains may differ in their size, shape, orientation and unit-cell dimensions. The variations among the domains will increase the angular range for each individual Bragg reflection, resulting in increased 'spot width'. The term mosaic spread is sometimes used to refer to the spot size, but more properly refers to the angular spread between domains, which increases the range of angles or 'rocking width' over which diffraction is observed. Variation in cell dimensions between domains or within a domain is sometimes referred to as strain. Strain also increases the rocking width, but can be distinguished experimentally from mosaic spread (Nave, 1998; Boggon *et al.* 2000; Kriminski *et al.* 2002).

Relatively few experiments to learn about larger-scale changes in lattice structure with cooling have been performed. Measurements of spot size and diffraction profile suggest large increases in both strain and mosaic spread when lysozyme crystals are cooled to 100 K (Nave, 1998; Kriminski *et al.* 2002). Experiments on cryo-cooled insulin crystals show approximately a five-fold increase in rocking width with cooling (Vahedi-Faridi *et al.* 2003). Topographic measurements on lysozyme crystals appear to lack the resolution to see domain structure after cooling (Kriminski *et al.* 2002). Thus, more work needs to be done on this topic.

The reduction of thermal motion with cooling could, in principle, improve the diffraction limit by increasing crystalline order. However, other factors usually dominate to decrease diffraction quality. Whether cooling procedures can be improved enough to allow the net reduction in thermal motion to actually extend the diffraction limit is an interesting and unanswered question.

3.3 Changes in the solvent

It is a characteristic of all macromolecular crystals that there are interstices between the macromolecules and that these regions are filled with solvent (Fig. 1). Because the unit cell usually shrinks more than the individual macromolecules, the interstices get smaller with cooling. The response of the solvent within these regions should thus play an important role in the effect of cooling on lattice order.

The size and shape of interstices varies from crystal to crystal. They may be isolated pockets or continuous channels running the length of the crystal. For crystals of some small proteins the interstices are very small and much of the solvent within them is associated with the surrounding macromolecules. More typically, the interstices are larger. Where a particular crystal lies on this continuum depends on the fraction of the crystal occupied by solvent, the size of the macromolecules making up the lattice and the particulars of the lattice packing. The solvent fraction

Table 1. *Packing parameters of some protein crystals*

Protein	PDB code	MW (kDa)	Solvent (%)	Channel diameter (Å)	Reference
Crambin	1CBN	5	30	9	Teeter *et al.* (1993)
Hen egg-white lysozyme	1LSE	14	41	16	Kurinov & Harrison (1995)
Concanavalin A	1JBC	25	47	30	Parkin *et al.* (1996)
Thermolysin	8TLN	35	49	18	Holland *et al.* (1992)
β-galactosidase	1F4A	465	58	20	Juers *et al.* (2000)
Acetylcholinesterase	1AX9	60	71	75	Ravelli *et al.* (1998)
Bacteriochlorophyll protein	4BCL	39	73	55	Tronrud & Matthews (1993)
VCAM-1	1IJ9	22	83	120	Taylor *et al.* (2001)
MobB	1P9N	16	90	190	Rangarajan *et al.* (2003)

A few protein crystals with their PDB codes and their packing parameters. MW is the molecular weight of the protein in the asymmetric unit. 'Solvent' is the volume fraction of the crystal occupied by bulk solvent and 'Channel diameter' is diameter of the largest solvent channel running the length of the crystal.

ranges from approximately 27 to 78% with values of ~43% being most typical (Matthews, 1968; Kantardjieff & Rupp, 2003). Comprehensive studies of interstice size and shape have not yet been carried out, but a brief survey of a few crystals showed a range of less than a few Ångstroms to greater than 100 Å (Table 1).

The wide range in interstice diameter suggests a varied solvent temperature response among different protein crystals. This issue was explored by Weik *et al.* (2001) who looked at ice formation in crystals slowly warmed from 100 K. Three protein crystals with different packing arrangements were used. Ice was more likely to form in the crystals with larger open solvent channels. Although there is likely to be a range of solvent behavior, in most crystals the solvent channels will be large enough for the solvent to show some bulk behavior. With respect to cooling one bulk property to consider is thermal contraction.

Figure 2 shows a schematic of three possible responses to the cooling of a crystal of a macromolecule. If the solvent contracts less than the interstices, then solvent will tend to be expelled from every unit cell throughout the crystal. If the crystal is cooled rapidly, or if the surface of the crystal freezes before the interior, this excess solvent will be trapped within the crystal. If the solvent shrinks more than the interstices, more solvent will tend to be imported from neighboring unit cells. This in turn will result in the formation of some type of voids within the crystal. If the solvent shrinks the same amount as the interstices, no solvent transport need occur, although rearrangement may be necessary to accommodate repacking.

When bulk solutions of cryoprotectant are cooled they contract and the amount of contraction depends on the cryprotectant concentration (Fig. 3). In contrast, when pure water is cooled to 100 K it expands by ~6·7%, whether it crystallizes or vitrifies (Ghormley & Hochandel, 1971). Adding cryoprotectant inhibits ice formation and promotes vitrification, but more importantly alters the amount of expansion. It has been shown with at least two crystal systems that there is an optimal cryoprotectant concentration which produces the best cooling results (Mitchell & Garman, 1994; Juers & Matthews, 2004). Additional experiments to measure solvent expansion suggested that at the optimal cryoprotectant concentration the amount of solvent extruded out of the interstices was minimized. As can be seen in Fig. 3, the amount of solvent contraction required for optimal cooling of β-galactosidase crystals appears to be ~7–8%. This occurs at 10% (v/v) higher for PEG 400 than for DMSO. Based at least on this example,

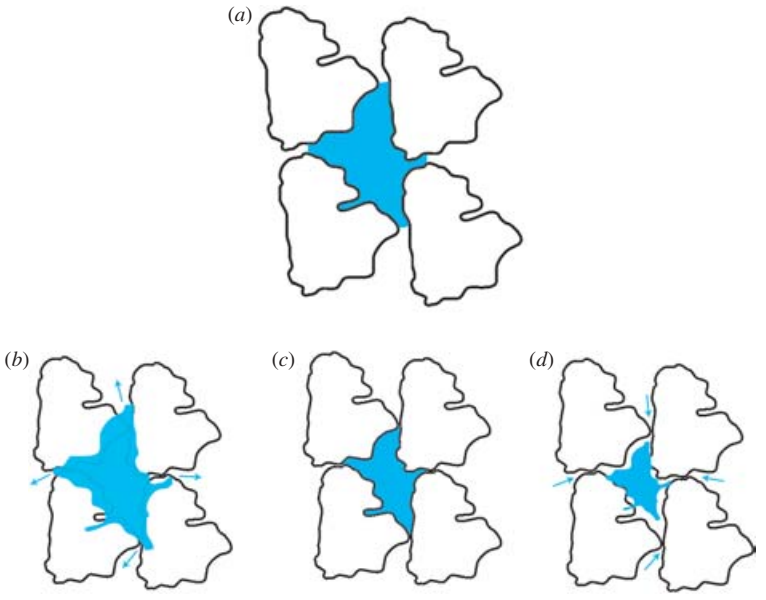

Fig. 2. Schematic showing three possible responses of solvent to cooling. (*a*) The room-temperature crystal, with the portion in blue illustrating the solvent occupying the interstitial space between the four macromolecules in the unit cell. (*b*)–(*d*) The crystal with its low-temperature packing arrangement, and three possibilities for solvent contraction. (*b*) The solvent shrinks less than the interstices, resulting in the extrusion of solvent into the neighboring interstices. (*c*) The solvent shrinks by the same amount as the interstitial space. Some solvent rearrangement is required to accommodate the lattice repacking. (*d*) The solvent shrinks more than the interstices, resulting in import of solvent into the interstice from neighboring ones.

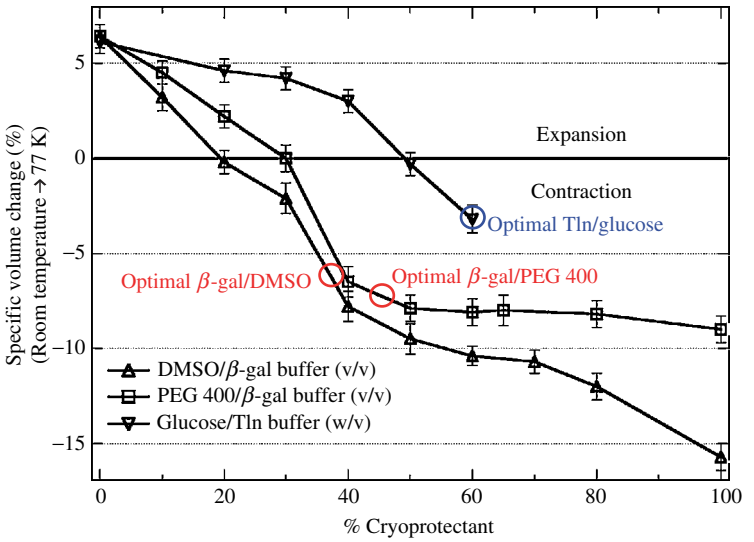

Fig. 3. Diagram showing the effect of cryoprotectant on solvent expansion/contraction. Three solvent systems are shown – that used with crystals of *E. coli* β-galactosidase with two different cryoprotectants (DMSO and PEG 400), and that for crystals of Thermolysin (Tln) with glucose as cryoprotectant (Juers & Matthews, 2004). The cryoprotectant concentration needed for optimal cooling of these crystals is noted with red (β-galactosidase) and blue (Thermolysin) circles.

optimization seems to depend on the change in volume of the cryosolvent and not on its chemical identity. As can be seen in the figure, at 60% (w/v) glucose, which is a saturating concentration, the solvent only contracts $\sim$3%. This would suggest that glucose is not a good cryoprotectant for crystals of β-galactosidase, since it cannot produce the required 7–8% contraction. Cryo-screening experiments have shown that cooling with glucose as a cryoprotectant for β-galactosidase crystals is only marginally successful.

It may seem curious that only a certain amount of solvent contraction preserves lattice order. One might imagine that the lattice could find acceptable packing for a range of solvent contractions. For example, if solvent contraction were equal to the macromolecular contraction, then the room-temperature lattice packing could be maintained. However, this appears not to be the case. One possible reason is that crystal-packing interactions which are optimal at room temperature need not be favored in the cold. As the crystal cools it can be energetically favorable for flexible side-chains, which are disordered at room temperature, to adopt well-defined conformations and form new lattice contacts. These new interactions may favor an alternative mode of crystal packing. Dao-pin *et al.* (1991) argued that if a pair of positively and negatively charged flexible side-chains each have two degrees of rotational freedom (e.g. χ_1 and χ_2) then the entropy cost of localizing these side-chains to form a salt bridge between them is $\sim$1·6 kcal mol^{-1} at room temperature. In going from 300 to 100 K this cost would be reduced by two-thirds. Given this reduction it is not surprising that alternative packing may be favored at low temperature. Insofar as is possible, the solvent must accommodate the alternative packing.

An example of an intermolecular contact in crystals of β-galactosidase that changes as a result of cooling is shown in Fig. 4. In this case the two macromolecules move 3 Å closer together and multiple interactions, not seen at room temperature, are present in the low-temperature crystal structure.

Direct measurement of solvent behavior within the crystal lattice is very difficult. Some information about the solvent can be gleaned from diffraction measurements. For example, sometimes after cooling a crystal, sharp rings appear, indicative of the crystallization of ice. However, such rings cannot, in general, distinguish between ice forming on the surface of the crystal, ice forming within the solvent on the surface of the crystal and ice forming within the solvent channels. More careful measurements need to be made to distinguish between these possibilities, such as correlating ice formation with unit-cell changes (Weik *et al.* 2001) or perhaps using a micro X-ray beam to compare different parts of the crystal.

Ice formation is easy to detect in the diffraction pattern and in general is to be avoided because the ice rings occlude diffraction spots. However, ice formation does not always mean poor diffraction; nor does poor diffraction mean ice has formed within the solvent channels. As described above, the more important parameter appears to be the amount of solvent contraction (Juers & Matthews, 2001, 2004; Kriminski *et al.* 2002). Thus, when searching for a cryoprotectant, one should explore a range of cryoprotectant concentrations other than the minimum to prevent ice formation in bulk samples of solvent. Both higher and lower concentrations can be explored. In the case of lower concentrations, the added use of oils will minimize ice formation in the external mother liquor.

4. The kinetics of cooling and lattice damage

So far the discussion has focused on thermodynamics. It appears that room temperature favors somewhat looser lattice packing while low temperature favors tighter packing. The contraction

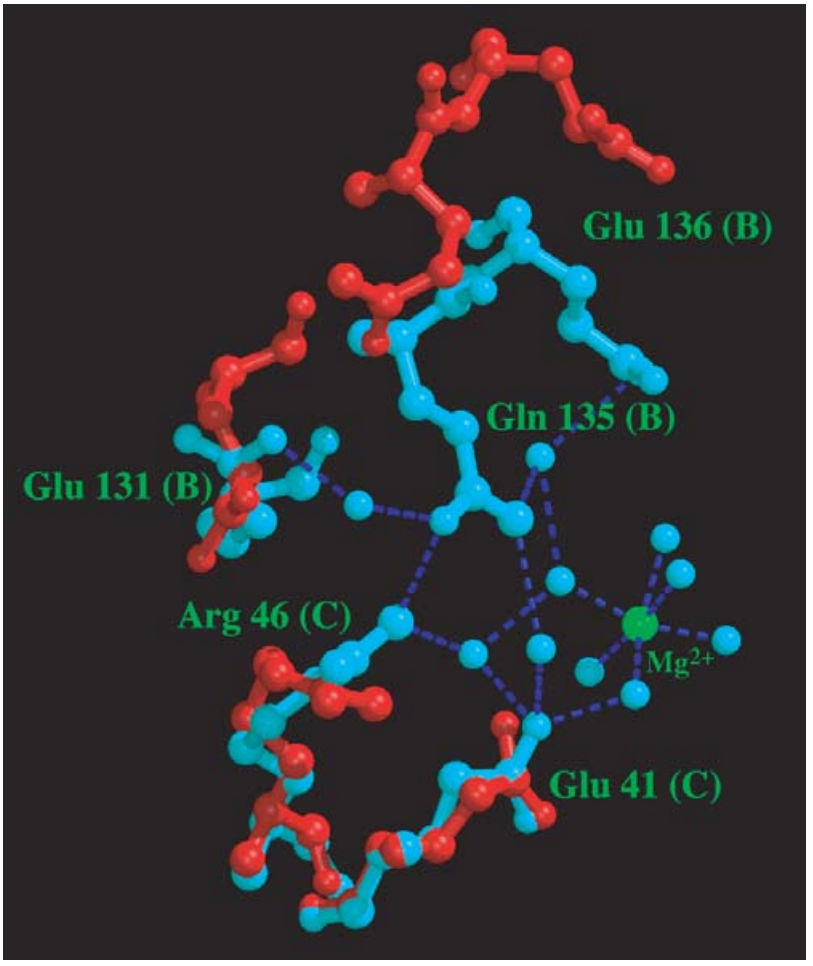

Fig. 4. An example of side-chain ordering at a lattice contact on freezing. Coordinates are from the refined models for room-temperature and low-temperature β-galactosidase. Atoms in the room-temperature structure are colored red and the low-temperature structure cyan. Side-chains in one molecule of β-galactosidase are labeled B and in the neighboring molecule C. At room temperature the five side-chains are poorly ordered with average B factors of $\sim$140 Å^2. On freezing the main-chain atoms move 3 Å closer together and the side-chains become ordered with average B factors of $\sim$30 Å^2. With the participation of solvent molecules and a putative Mg^{2+} (in green), they generate a region of crystal contact much more extensive than that seen at room temperature. (From Juers & Matthews, 2001.)

can be understood in terms of decreased thermal motion with decreasing temperature. This contraction occurs for the macromolecules themselves but is more dramatic in the case of the lattice contacts. This is because the surface side-chains are more mobile than those in the interior of the protein. When these motions are frozen out at low temperature, the intermolecular contacts can 'tighten up' more than the intramolecular ones.

As discussed above, under ideal conditions the solvent contraction will match the contraction of the interstices. Otherwise, solvent needs to be transported into or out of the unit cell. In cases where the interstices form isolated pockets of solvent, transport may be impossible and unless the solvent contracts the correct amount, lattice damage may result. In cases where the interstices are connected via channels, there is greater opportunity for solvent transport. In such cases the

solvent could, in principle, be completely transported in or out of the crystal. In this situation it might seem that slow cooling would be preferred, allowing solvent transport to occur on a time-frame that would not damage the crystal lattice. However, slower cooling usually favors ice crystal formation, which would, at minimum, alter the contraction of the solvent and could itself damage the crystal. Thus, the best cooling rate may be the slowest rate that causes vitrification and prevents ice formation. This rate depends strongly on the cryoprotectant type and concentration (Sutton, 1991). Higher cryoprotectant concentrations may permit slower cooling rates to be used without ice formation, giving the lattice a chance to repack and solvent transport to be completed. The disadvantage to higher cryoprotectants is that usually the viscosity increases, which inhibits lattice repacking and solvent transport. It also seems likely that with crystal packing in which the solvent channels are very small, slow cooling may also be possible without ice formation, since with these lattice configurations, the solvent may not show bulk behavior. Although it seems likely that the cooling rate is important, there have been no systematic studies of its effect on low-temperature lattice order, partly because it is difficult to control.

Often, however, cooling must occur rapidly in order to prevent ice formation. The distance the solvent can be transported through the solvent channels during cooling should depend then on several factors – the openness of the channels, the viscosity of the solvent, and how quickly vitrification occurs. Solvent transport is driven by the combined effects of macromolecule shrinkage, lattice repacking, and solvent density change, all of which are triggered by the drop in temperature. Solvent transport is precluded when the solvent vitrifies, which is also triggered by the drop in temperature. A good cryoprotectant, then, would be one which causes the bulk solvent to go through its density change long before vitrification occurs.

In many cases the solvent can presumably only be transported a small distance through the crystal during cooling. In the case of extrusion, Kriminski *et al.* (2002) suggest that the solvent is transported out of small domains in which the lattice remains well ordered, and collects in solvent-rich regions scattered throughout the crystal. These may be inherent weaknesses and voids in the room temperature crystal, or they may be caused by the solvent extrusion itself. This should increase the angular separation between neighboring domains, increasing spot widths in the diffraction pattern. Different domains may have different success at extruding solvent, which might increase the spread in cell dimensions, and perhaps domain orientation, also increasing spot widths.

In the case of solvent importation, the weaknesses and voids may act as solvent reservoirs. Lacking these reservoirs the lattice will be forced to collapse at least to some degree. Different unit cells or domains may have different success at importing solvent. This will result in a larger spread of cell dimensions, and, consequently, increased spot widths.

The two types of lattice damage described above are somewhat different. The first is characterized by a spreading apart of molecules within the unit cells, unit cells within the domains, and domains within the crystal, while the second is characterized by collapse and contraction at these different length-scales. As will be discussed below, we have found that the latter type of lattice damage appears to be more reversible than the former type.

At this point the experimental evidence for the importance of solvent extrusion or importation is indirect. For example, X-ray topographs appear to show small domains within lysozyme crystals after cooling (Kriminski *et al.* 2003). Also solvent-density measurements indicate that the optimal cryoprotectant concentration is that which corresponds to minimal solvent transport during cooling (Juers & Matthews, 2004). Improved topographic methods, or possibly electron microscopy, may help to answer some of these questions.

Even if the solvent composition for optimal contraction is chosen, cooling may not work perfectly. Experiments have shown that even at the optimal cryoprotectant concentration, mosaicity often increases dramatically. In the case of *E. coli* β-galactosidase, mosaicity increases of approximately five-fold were observed (Juers *et al.* unpublished observations). Occasionally, much smaller mosaicity increases have been observed, but this does not happen frequently enough for an understanding of the necessary conditions for such a result.

The reason for such dramatic increase in mosaicity with cooling even at the optimal cryoprotectant concentration is unclear. One possibility has to do with the timing of the events during cryo-cooling. As mentioned above, lattice repacking and solvent transport both require solvent rearrangement, which is impossible after solvent vitrification. All of these events are ultimately driven by the temperature change, so there is an issue of timing. If the viscosity increases greatly before the repacking and solvent transport are completed, then the crystal will be trapped in a disordered state. The kinetics of repacking, solvent-density change, solvent transport and vitrification has not yet been investigated.

Another possibility to explain the mosaicity increase is inhomogeneous cooling. When a crystal is cooled in a cold gas stream, heat transport occurs via conduction through the crystal and convection across the boundary layer between the crystal and the cooling medium. Which process is rate-limiting depends on the conditions. Calculations suggest that for medium to smaller crystals cooled in a gas stream, convection is the slow step, while for larger crystals, especially those cooled in a non-boiling liquid, conduction is the slow step (Kriminski *et al.* 2003). If conduction within the crystal is rate-limiting, then inhomogeneities in the temperature gradient within the crystal might cause strain. Thus, faster cooling may only be advantageous to a point, after which increased cooling rates might actually be damaging to lattice order.

5. Annealing

A recent development in cryo-cooling techniques is the possibility of recovering 'lost' diffraction by cycling crystals between the low temperature and some higher temperature. At least three variations on this annealing technique that have been described.

In the first approach, the crystal is left in the loop on the goniometer while the cold stream is blocked for a certain period of time and the crystal allowed to warm up, possibly to room temperature (Yeh & Hol, 1998). The crystal is left at the warmer temperature for a few seconds and then recooled. In another variation, after the cold stream is blocked, the crystal is removed to a drop of mother liquor for several minutes before remounting and recooling (Harp *et al.* 1998). In the third variation, the crystal is left in the loop, but the warm temperature is restricted to 230–250 K rather than room temperature (Kriminski *et al.* 2002).

Recent experiments have shown that when the cold stream is blocked and the crystal warms up, water can be transported into or out of the crystal. The amount of water transport depends on several factors including the humidity of the air in the vicinity of the crystal (Juers & Matthews, 2004). This water transport can play a major role in the first type of annealing described, because the contraction of the bulk solvent changes as the water content is altered. Solvent which contracted too much in the first cooling cycle, may contract the correct amount in a later cycle once its composition has changed to give the correct thermal contraction.

This behavior is illustrated in Fig. 5 (Juers & Matthews, 2004). A crystal of β-galactosidase, initially at $\sim$35% (v/v) DMSO, somewhat higher than optimal, was flash-cooled. The stream of dry nitrogen gas keeping the crystal frozen was then interrupted for $\sim$3 s, allowing the crystal to

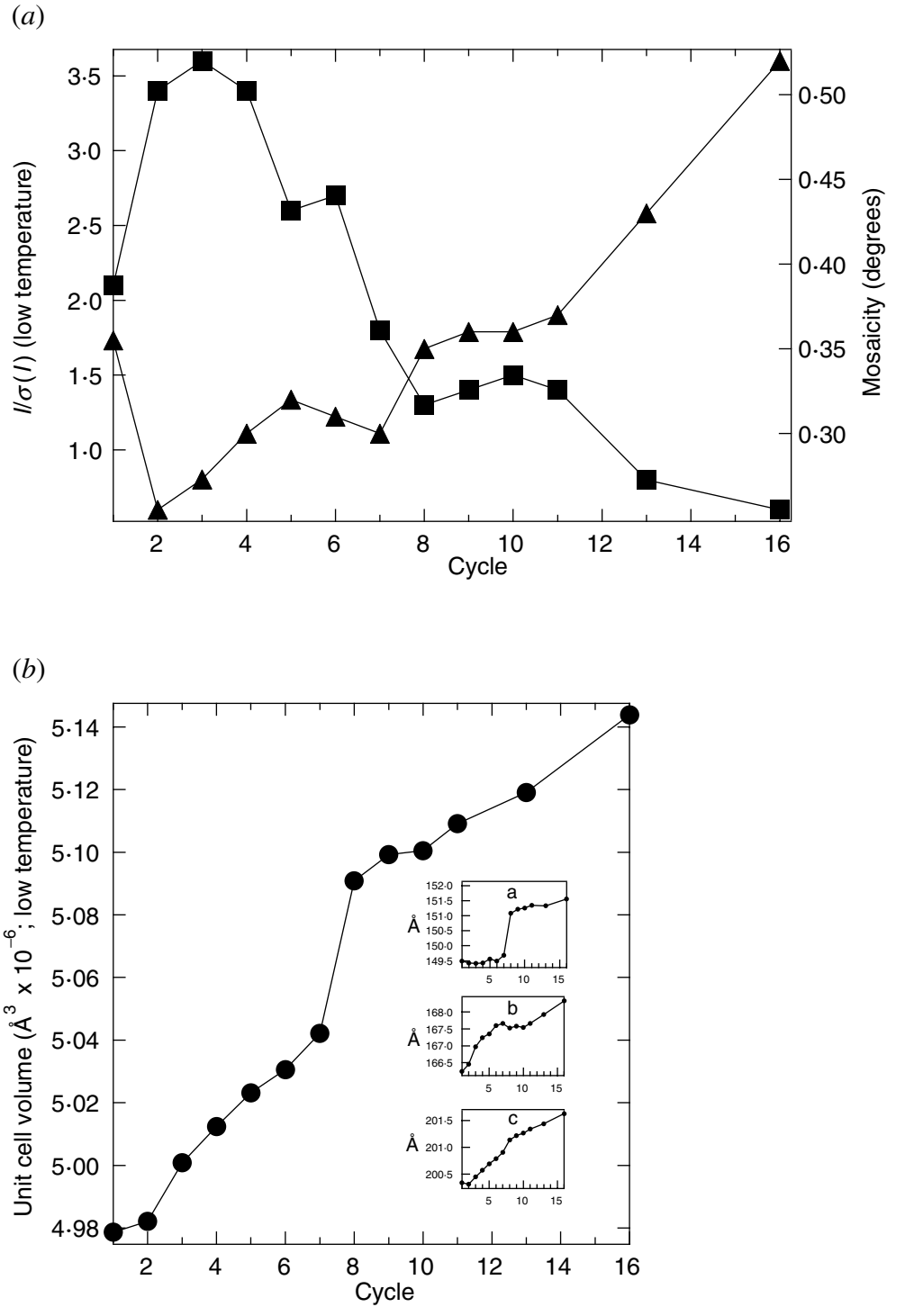

Fig. 5. Evolution of crystal parameters over 16 cycles of low-temperature/room-temperature cycling of a crystal of β-galactosidase with DMSO as a cryoprotectant, starting at $\sim$35% (v/v). (*a*) Change in mosaicity (▲) and $I/\sigma(I)$ (■) for data to 2·3 Å resolution. (*b*) Changes in unit-cell volume and, in the inset graphs, the individual unit-cell parameters. (From Juers & Matthews, 2004.)

warm up in the ambient environment. Although the warming-up period is brief it allows some condensation of water vapor on the crystal. As the temperature rises the cryosolvent within the crystal becomes a liquid and the water on the surface equilibrates through the crystal. This in turn dilutes the cryosolvent so that its volume decreases less on cooling (Fig. 3). During subsequent cycles of warming and cooling the uptake of solvent is reflected in an increase in the volume of the unit cell. Note also that there is an essentially discontinuous change of $\sim$1·3 Å in the *a* cell edge at about cycle 8, reflecting a distinct change in crystal packing. Note also that the quality of the diffraction pattern, as measured by $I/\sigma(I)$ (Fig. 5) initially improves until the optimal concentration of cryosolvent is reached, and then gradually deteriorates.

Figure 6 shows another experiment illustrating the effect of moving water in and out of the crystal during the melting phase. A crystal of β-galactosidase, initially at 35% (v/v) DMSO was flash-cooled. The stream of dry nitrogen was then interrupted for a few seconds, thawing the crystal either in a dry or humid ($\sim$50% RH) atmosphere. Figure 6*a* shows that in the humid atmosphere, the unit-cell volume increases, while in the dry atmosphere it decreases. Further, Fig. 6*b* shows that data quality can initially improve whether the atmosphere is humid or dry, but then it deteriorates after a few cycles, suggesting that the crystal is being moved into and out of its optimal cryoprotectant concentration. The overall degradation of the diffraction suggests that the reversible effects are also accompanied by gradual irreversible damage to the lattice. To a certain extent, '*in situ*' annealing can recover 'lost' diffraction in crystals of β-galactosidase either by solvent transport into the crystal, in the case of too high cryoprotectant, or solvent transport out of the crystal, in the case of too low cryoprotectant. Generally, though, the above experiments showed that '*in situ*' annealing was more successful when the initial cryoprotectant concentration was too high rather than too low. In the former case, the solvent initially contracts too much, and the lattice tends to collapse with cooling. This type of lattice damage appears to be more reversible than when the cryoprotectant concentration is too low.

Often the search for a suitable cryosolvent focuses on finding the minimum cryoprotectant concentration to prevent ice formation. Generally, this is because equilibration to higher cryoprotectant concentrations requires additional experimental effort and, in any event, may be damaging to the crystals. However, if the crystals are robust to equilibration to higher cryoprotectant concentrations, we suggest it may be better to err on the side of too-high cryoprotectant concentration than too-low.

There appear to be factors involved in annealing other than the adjustment of solvent content. In the second type of annealing described above, the warmed crystal is bathed in a drop of mother liquor, during which it presumably re-equilibrates to the original solvent condition. Thus, in this case it seems likely that some improvement in diffraction quality can be achieved even though the bulk solvent ultimately retains the same composition during repeated cool–melt cycles.

In the third type of annealing, the crystal is warmed only to 230–250 K for a few seconds to a few minutes, and then cooled again to 100 K (Kriminski *et al.* 2002). In these experiments, measurements were made to distinguish between strain and mosaic spread. Most of the benefit from annealing was due to a decrease in strain, while the mosaic spread was mostly unaffected. The release of strain was attributed to increased local mobility of water at $\sim$243 K. Because this is far below the freezing point of bulk water, reorientation of domains is less likely since this will probably require larger-scale water transport. For the same reason there is probably not a general change in interstitial solvent composition and associated contraction. This annealing seems to be dominated more by local effects.

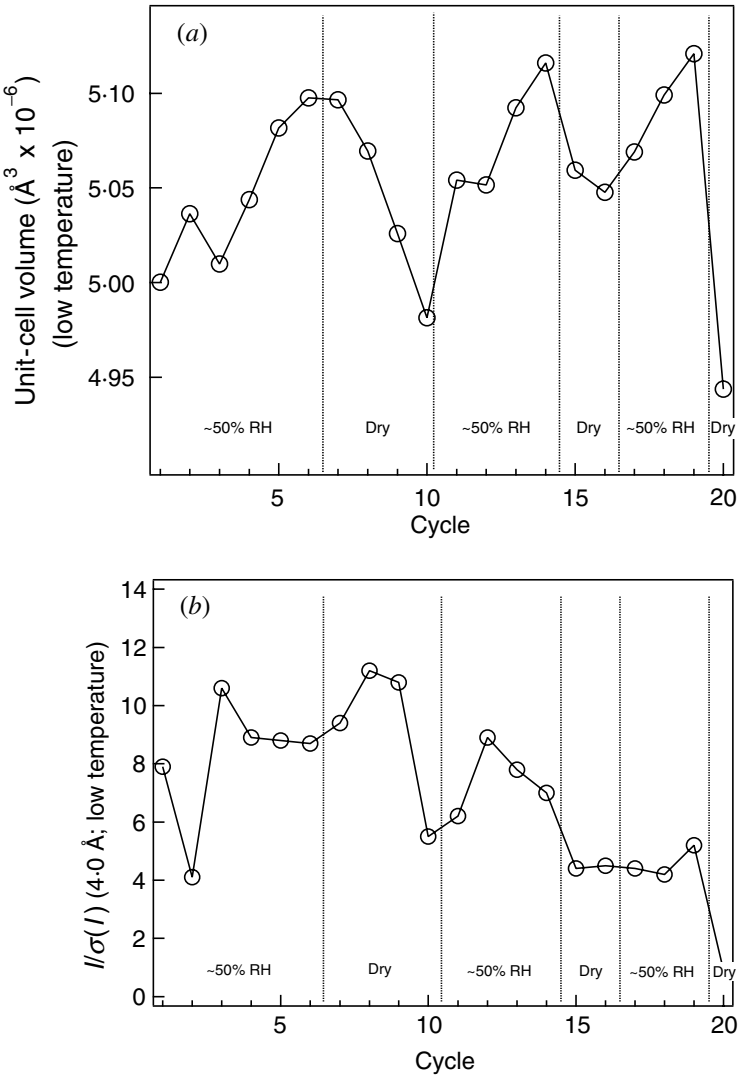

Fig. 6. Evolution of crystal parameters for a crystal of β-galactosidase subjected to low-temperature/room-temperature cycling with different humidity conditions. The crystal was equilibrated to 35% (v/v) DMSO (slightly above the optimal of 30%) prior to the experiment. (*a*) Changes in unit-cell volume for a crystal subject to cooling and thawing in an 'ambient' atmosphere [$\sim$50% relative humidity (RH)] and then in a dry atmosphere. (*b*) Plot showing that data quality, as measured by $I/\sigma(I)$, can initially improve and then deteriorate when the crystal is subject to cooling and thawing in either the humid or dry atmosphere. (From Juers & Matthews, 2004.)

An unanswered question is whether there are some crystal systems which require multiple cooling cycles to reach the preferred lattice packing. That is, with the first cycle, the crystal only gets part of the way to the optimal low-temperature conformation because it is limited by kinetic effects such as solvent vitrification before repacking is completed. When the crystal is warmed it may not revert completely to its original room-temperature conformation, and on re-cooling may gradually progress toward optimal repacking.

6. Biological relevance at 100 K

Most crystal structures being reported today are determined at cryogenic temperature (Garman, 2003). The lattice repacking that usually accompanies the transition to low temperature is indicative of an alteration in intermolecular interactions as a function of temperature (Juers & Matthews, 2001). This is not to say that intermolecular forces are fundamentally different at 100 K *versus* 300 K. They are not. It is to say, however, that biologically relevant interactions are often mediated by flexible amino-acid side-chains on the surfaces of macromolecules, and such interactions may be sensitive to temperature. Certain flexible surface side-chains may be mobile at room temperature, may not be seen in room-temperature crystals, and may be biologically irrelevant. In a low-temperature crystal structure the same surface side-chains may be well-ordered and may participate in well-defined interactions (Fig. 4). The problem is to decide whether such apparently well-defined interactions are biologically significant.

A thoughtful analysis of these issues showed that structures based on low temperature X-ray diffraction are not of molecules at thermal equilibrium (Halle, 2004). This analysis suggests that when the temperature is lowered during cooling, the many degrees of freedom in the macromolecule are quenched at $\sim$200 K. At this temperature, equilibria may be shifted towards low-enthalpy states, such as side-chain conformation switching, weak ligand binding and water-molecule binding to non-polar cavities. It was noted that many complicated solvent networks, such as 5- to 7-membered water rings, appear to be cryo-artifacts. That is, when the temperature is increased, these structures disappear in the crystallographic electron density maps.

Care should therefore be taken not to over-interpret electron-density maps based on diffraction at low temperature. However, the other side is that many biologically relevant protein–ligand interactions are weak and visualization of these types of interactions is difficult. It is possible that the low enthalpy, weakly bound states trapped with cryo-cooling are exactly the room-temperature transient states that need to be observed. Knowing that they are such is another matter.

7. Acknowledgments

This work was supported in part by NIH grant no. GM20066 to B.W.M.

8. References

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