‘Cool’ crystals: macromolecular cryocrystallography and radiation damage

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Macromolecular crystals commonly suffer rapid radiation damage during room temperature X-ray data collection. Therefore, data are now routinely collected with the sample held at around 100K, significantly reducing secondary radiation damage, and usually resulting in higher resolution and better quality data. At synchrotron sources, the frequent observation of radiation damage even at cryotemperatures has prompted the development of exciting new experiments aimed at characterising and reducing this damage, and using it for structure determination and enzymatic studies. Current research into cryotechniques seeks to understand the basic physical and chemical processes involved in flash-cooling and radiation damage, which should eventually enable the rational optimisation of cryoprotocols.

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Current Opinion in Structural Biology 2003, **13**:545–551

This review comes from a themed issue on
Biophysical methods
Edited by Brian T Chait and Keith Moffat

0959-440X/$ – see front matter

DOI 10.1016/j.sbi.2003.09.013

Abbreviations

MAD	multi-wavelength anomalous dispersion
PDB	Protein Data Bank
RIP	radiation damage-induced phasing
SAD	single-wavelength anomalous dispersion
S/V	surface area/volume

Introduction

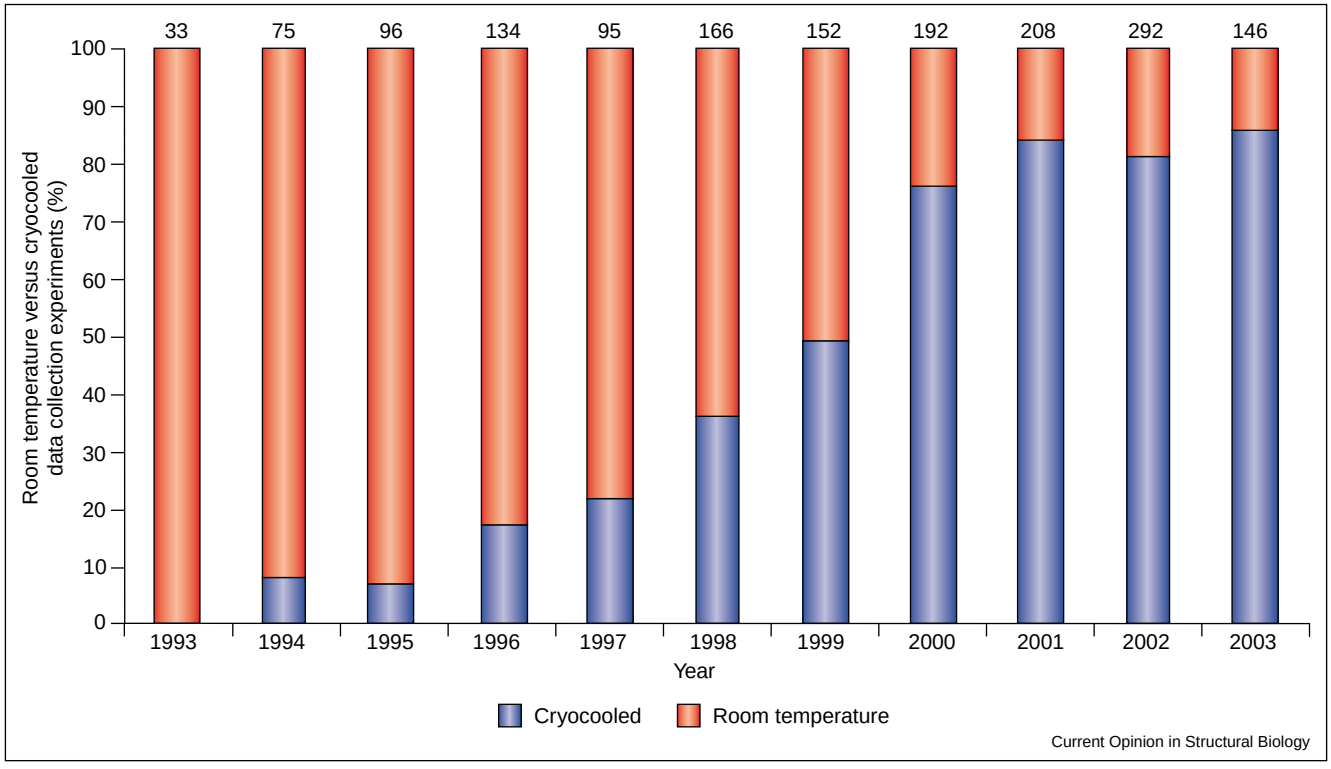
Most macromolecular crystal diffraction data are now collected at or near 100K [1•,2•,3] (Figure 1). The key advantage of this technique is the enormous reduction in secondary radiation damage to the sample during X-ray data collection compared to data collection at room temperature. This is because, at cryotemperatures, most of the free radicals produced by the incident radiation have much lower diffusion rates so they do not travel as far through the crystal, thus avoiding as much damage. The prolonged crystal life-times usually result in better quality and higher resolution data. Other benefits include the facility to flash-cool crystals into cryogen and store them while they are in peak condition, and the potential for using cryocrystallography in new experiments, particularly the freeze-trapping of reaction intermediates. Some of the disadvantages of cryocrystallography are that cryoconditions have to be established, a reliable cryostat must be installed and the crystal mosaicity usually increases on flash-cooling.

Nevertheless, even in samples that are cryocooled, observation of radiation damage has become commonplace at undulator synchrotron beamlines (Figure 2). It is a limiting problem in the optimum use of these high brilliance beams, which are routinely attenuated or defocused. Radiation damage also accounts for the increasing popularity of single-wavelength anomalous dispersion (SAD) experiments, as the dispersive signal necessary for success in multi-wavelength anomalous dispersion (MAD) experiments is easily obscured by nonisomorphism arising from radiation-induced unit cell expansion and specific structural changes [4–6]. Thus, in order not to compromise the phasing information from MAD, care should be taken not to over-irradiate crystals; a well-conducted MAD experiment will certainly provide more phasing information than a well-designed SAD experiment. Unless the effects of radiation damage on a structure are understood, incorrect conclusions about the biological mechanism can be reached, in particular due to the decarboxylation of residues.

For a crystal held at 77K, the theoretical X-ray dose limit required for the crystal to lose roughly half its diffraction power has been calculated to be $\sim 2 \times 10^7$ Gy [7]. For a typical protein crystal, this dose is delivered by 1.6×10^{16} ph/mm^2 incident 1.54 Å X-rays; this is equivalent to about 2.5 years on a state-of-the-art rotating anode and a few minutes on an undulator beamline. Experimental observations indicate that the latter limit holds. Samples containing heavily absorbing atoms will have a higher morbidity, as they reach the dose limit in a shorter time due to their increased mass absorption coefficient. Software now available (http://www.esrf.fr/exp_facilities/ID14-4/ID14-4.html) will allow experimenters to calculate the theoretical time to the dose limit, using information on the crystal content (protein and buffer) and the incident beam conditions, and thus to plan their experiments accordingly.

The focus of recent research into cryotechniques has moved from establishing empirical conditions and suitable crystal-mounting hardware to studies of the physical and chemical processes underlying the cooling methods and the radiation damage suffered at cryotemperatures;

Figure 1



The percentages of room temperature and cryotemperature data collection experiments from a survey of all papers published in *Acta Crystallographica D* from January 1993–May 2003 (the number of experiments is shown at the top of each bar). The trend is clear, although the increase in the proportion of cryotemperature experiments reported in the journal lags behind that reported in [10]. The PDB could not be used for this survey, as data collection temperature is not required information and is thus missing from most depositions.

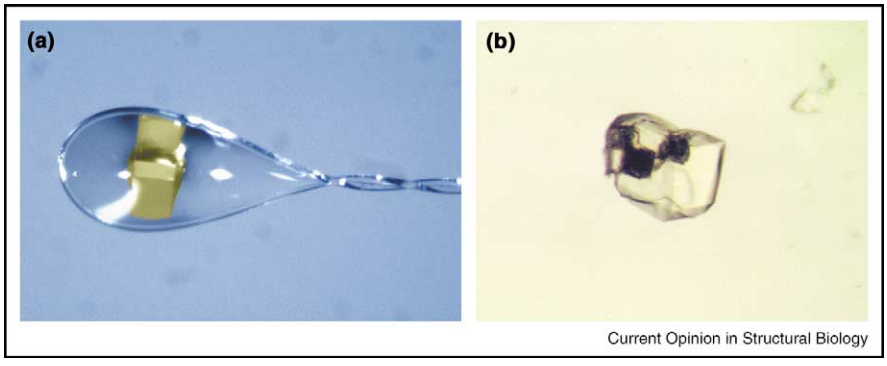
the importance of the latter is now being recognized by the structural biology community.

Optimisation of cryotechniques

Currently, mounting a crystal for cryocrystallography involves suspending it by surface tension in cryobuffer within a <1 mm diameter loop [8] of thin fibre (Figure 2) and then flash-cooling it in gaseous or liquid cryogen. The cryobuffer is designed to vitrify and prevent crystalline ice formation.

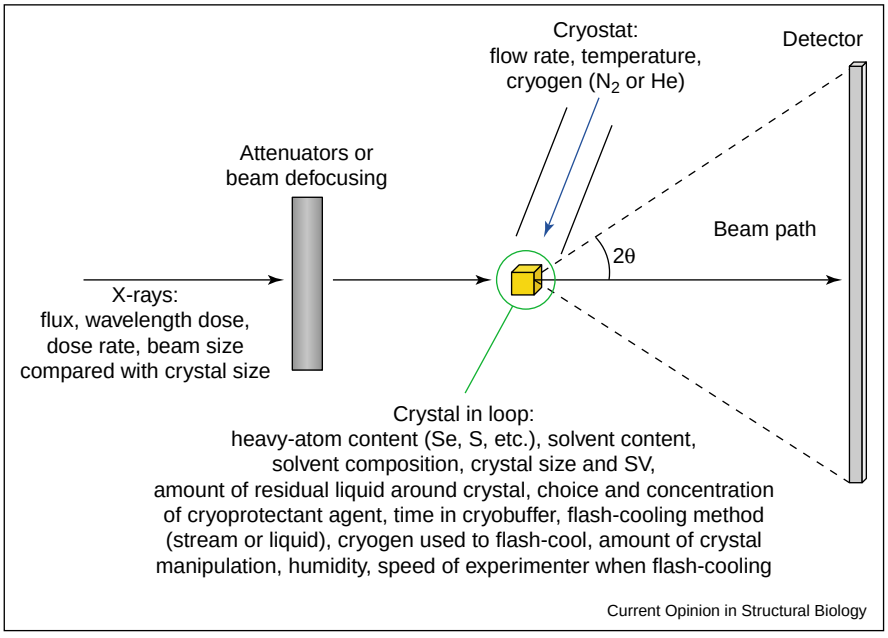
The parameters affecting cryocrystallography experiments can be divided into two classes. First, the physical and chemical environment of the crystal in the loop,

Figure 2



Cryocrystallography and radiation damage. **(a)** A 100K cryocooled protein crystal held in a vitrified cryobuffer loop. **(b)** A 100K cryocooled protein crystal that has been subjected to data collection at three positions on a synchrotron undulator X-ray beamline and then allowed to warm up in cryobuffer. The release of secondary radiation products on warming has blackened the crystal at the beam spots. Additionally, gas evolved on warming.

Figure 3



Diagrammatic representation of an X-ray diffraction experiment, annotated to show the parameters believed to be relevant to cryocooling outcomes and radiation damage progression.

including choice and concentration of cryoprotectant agent, solvent content of the crystal, size of the crystal and surface area/volume (S/V) value of the crystal. Second, the external variables, including the cooling regime — the cryogen used for flash-cooling, the cryostat cryogen (nitrogen or helium), the cryogen flow rate and the temperature used during data collection — and the incident beam conditions of dose, dose rate, flux and wavelength (Figure 3).

The effect of some of these factors has recently been theoretically analysed and the variables placed in order of relative importance, as follows: crystal solvent content, solvent composition, crystal size and shape, amount of residual liquid around the crystal, cooling regime and, least important, the choice of cryogen [9••]. This study forms a sound basis for systematic experimental tests to find optimised cryoprotocols.

Physical and chemical environment of the crystal

Crystal mounting

The loop method of mounting has now almost entirely replaced earlier cryomounting techniques and hardware for cryocrystallography is available from several commercial vendors. The detailed implementation of the common basic ideas varies and unfortunately the systems are not cross compatible, as standard dimensions (pin length, magnet diameter, etc.) are yet to be established. Current developments are focused on accommodating cryocooled crystal-mounting robot cassette requirements. Improved tools should result, especially in regard to storage, transport and retrieval of crystals, for which the success rate is often disappointingly low. Robots for mounting precooled crystals from liquid nitrogen storage Dewars onto beamline goniometers are being tested at most synchrotron sites, and are already proving their worth in speed and reproducibility.

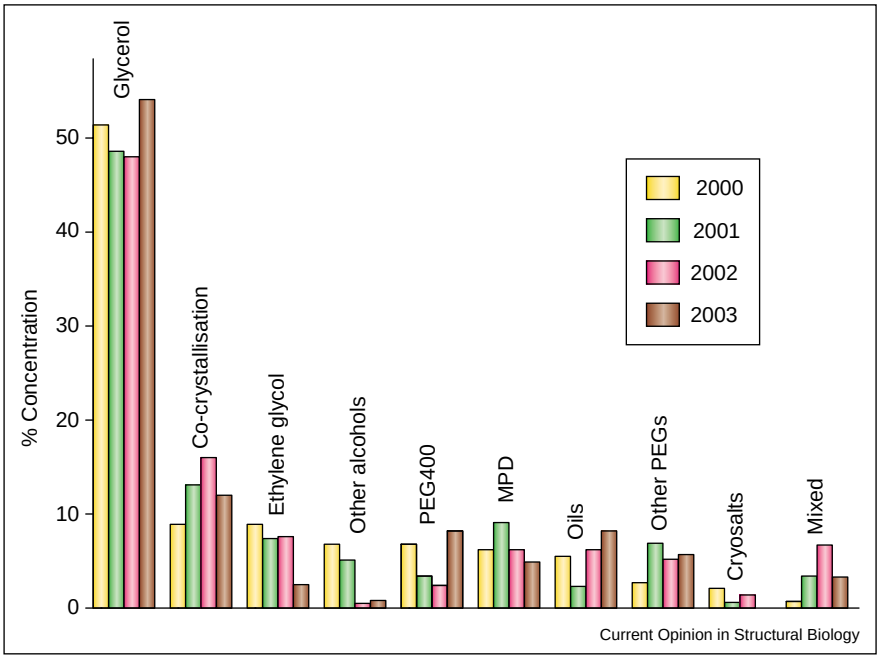
Cryoprotection

Although there is quite a choice of cryoprotectant agents [2•,10], a survey (Figure 4) shows that glycerol, mainly at between 20–25% concentration, is the most popular. This does not mean that it is the best cryoagent; most experiments involve little optimisation of cryoprotocols and glycerol is often just the easiest available solution that will 'do the job'.

The vitrification quality of cryoprotectant solutions has been quantified using the standard deviation of the derivative of the intensities of the X-ray scattering rings with respect to resolution. The concentrations of glycerol, PEG400, ethylene glycol and 1,2 propanediol necessary to cryoprotect Hampton Screens I and II [11] have thus been tabulated, providing a useful resource.

The optimum scenario, however, is to grow the crystal in a solution that is already cryoprotected. This strategy has been given added impetus by the moves to high-throughput crystallography, for which it is highly desirable to reduce the number of crystal manipulation steps.

Figure 4



Bar chart showing the most commonly used cryoprotectant agents. Co-crystallisation refers to conditions that do not require the addition of cryoprotectant agent. Cryobuffers that contain more than one cryoprotectant were grouped into the 'mixed' category. Data were compiled from a survey of all papers published in *Acta Crystallographica D*: 2000, 146; 2001, 175; 2002, 210; Jan–May 2003, 122. MPD, 2-methyl-2,4-pentanediol.

To this end, more commercial screens designed with cryoprotection in mind are being used with crystallisation robots.

The role of the cryobuffer in the 2–7% unit cell contraction usually observed upon cryocooling a protein crystal, as well as in the change in the area of the protein involved in lattice contacts, has been analysed in detail. Tenuously interacting intermolecular interfaces are altered on cooling, with the interaction areas of glutamic acid, arginine and glutamine increasing the most. Therefore, caution should be exercised when interpreting cryocrystallographic data on protein–protein and protein–nucleic acid interactions [12•]. These results support the argument for the data collection temperature to become compulsory information upon deposition of structure coordinates in the Protein Data Bank (PDB).

Crystal size

Due to faster heat transfer, small crystals and crystals with a large S/V are generally easier to flash-cool than larger ones, and exhibit a smaller increase in mosaicity. Larger crystals, however, have a greater diffracting power and will yield more useable diffracted photons before reaching their dose limit. There is thus a minimum crystal size that can be expected to give a data set at a specified resolution for a certain unit cell [6,13–15].

External variables

Cryogen temperature for data collection

Currently, a gaseous nitrogen stream held at 90–110K cools the crystals during data collection. Open flow helium is an alternative cryogen that can reach lower temperatures (down to ~16K). Theoretically, lower temperatures should enhance high-resolution diffraction due to the reduced B-factors [3]. Experimentally, there are some indications that this may be the case, with higher resolution, improved data quality and extended life-times being reported [16,17], although these results have not been reproduced elsewhere; certainly, the signal-to-noise can improve. There are pertinent cost implications if helium were to be adopted as the preferred cryogen at synchrotrons and so far the experimental evidence has not been entirely persuasive. Nevertheless, helium has a larger heat transfer coefficient than nitrogen and, even at 100K, vitrification during flash-cooling is faster. Thus, less cryoprotectant agent is required and, for cases where no satisfactory cryoprotocol can be found, helium is worth trying.

Solvent behaviour in crystals as a function of their temperature has been elegantly characterised and the vitreous to crystallization ('glass') transition temperature determined for two crystal forms of acetylcholinesterase, one with larger solvent channels than the other [18•]. From this study, it is clear that protein crystals remain in a metastable

state below 155K, setting an upper limit for adequate cryotemperatures. This result has been confirmed by a study of human lysozyme at seven temperatures between 113K and 178K. Above 150K, normal mode analysis showed a sharp increase in dynamic behaviour [19].

Thus, the crystal temperature should always be kept below 155K, except during annealing (slow warming of cooled crystal to room temperature and then re-flash-cooling). Annealing can sometimes result in improved resolution for treated crystals and can be accompanied by a decrease in mosaicity as mosaic blocks realign [17]. Warming hen egg-white lysozyme crystals to between 230K and 250K, and then re-flash-cooling them gave more reproducible results than warming to room temperature; this may be a generally applicable strategy [20•]. The cryocooling and annealing process has been thermally imaged using an IR camera [21•]. Although these thermal measurements are so far qualitative, when calibrated this technique promises to be a useful new tool for fundamental cryocrystallography research.

Cryogen used for flash-cooling

In protein crystallography experiments reported in *Acta Crystallographica D* during 2002, 60% of crystals were flash-cooled in a nitrogen gas stream (~100K), 37% in liquid nitrogen (77K) and 3% in liquid ethane or propane; experimentally, there is no compelling evidence to favour the last choice. Recently, there has been a welcome demise in propane use because of the extra handling precautions necessary and the difficulties of safely shipping it in Dewars. The theoretical analysis mentioned above [9••] concludes that cryogen choice is the least important variable.

Crystal heating

Synchrotron X-ray beam heating causes a rise in crystal temperature, and has been calculated by finite element analysis [22] and by analytical methods [23]. The latter study concluded that, for a 100K nitrogen cooling regime and a 100 μm thick crystal, the sample temperature rise for a flux of 10^{13} ph/s/mm^2 would be about 6K at equilibrium, lower than was previously thought. In an improved and extended theoretical analysis [9••], these results were confirmed for small crystals, but for larger crystals held at 100K in the highest brilliance beams, heating of 20K was predicted. Experimentally, no evidence of a significant temperature rise was observed up to 4×10^{12} ph/s/mm^2 by lattice expansion measurements on an organic light-atom crystal [24]. If, however, the crystal heats to above 155K, the protein local conformational flexibility increases and the rate of radiation damage drastically accelerates [25•].

Dose/dose rate effects

Experiments to characterise the relationship between damage progression and dose have shown it to be linear up to 1×10^7 Gy [13]. This upper dose limit seems to hold between 40K and 150K [16]. It is postulated to be the dose at which secondary and tertiary damage becomes important, a suggestion prompting lively current debate. Recent experiments searching for a dose/dose rate effect by analysis of data reduction statistics indicated that damage depends only on absorbed dose and found no evidence of any dose rate effect up to 10^{15} ph/s/mm^2 [15], in other words, the current maximum flux on an unattenuated third-generation undulator beamline. For holoferritin, however, which has a highly absorbing iron core, a dose rate effect has been reported at lower fluxes, attributed to the calculated crystal temperature rise of ~100K [26].

Towards controlling radiation damage at cryotemperatures

Radiation damage control strategies are very limited. Crystals soaked in heavy-atom compounds can be back soaked to remove disordered absorbers and consideration can be given to exchanging heavy components in the mother liquor.

There is currently no easy way to monitor damage on-line, so mitigation strategies are hard to test quantitatively other than by looking at the rate of specific structural damage. Unfortunately, the rate of unit cell expansion, although linear with dose, cannot be reproduced among different crystals of the same protein [26,27].

A promising area is the use of scavengers; the existence of specific structural damage at 100K shows that some radicals are still mobile in the crystal. Thus, free-radical scavengers may be able to react with these species and reduce their mobility and reactivity. Analysis of electron density maps, coupled with monitoring the formation of a disulfide-related radical species with an off-line microspectrophotometer, showed that co-crystallization with 0.5 M sodium ascorbate was effective in protecting lysozyme [27].

Correcting data for radiation damage

Researchers are becoming increasingly aware of the effects of radiation damage on the biological conclusions being drawn from crystallographic structures. A study of the primary photoreaction of bacteriorhodopsin, in conjunction with an on-line spectrophotometer (350–800 nm), showed that a synchrotron X-ray beam induced half the protein to convert into an orange species during 100K data collection with an incident total dose of 5×10^{14} ph/mm^2; further experiments at different radiation doses were necessary to identify the true structural changes [28].

Software correction procedures for extrapolating individual reflection intensities back to their zero dose level are currently being developed [29], using multiple

measurements of each reflection. This strategy should enable the useful life-time of the crystal to be extended. It is anticipated that these ideas will eventually be incorporated into the standard data processing software.

Utilising radiation damage

The specific structural damage inflicted by X-rays has been used to advantage in elegant experiments that establish a new way of phasing macromolecular structures, suitable for use at high brilliance synchrotron beamlines. In the radiation damage-induced phasing (RIP) method, a low dose (with attenuators) data set is collected, followed by a 'burn' of approximately half the Henderson dose limit. This dose destroys the disulfide bonds and causes other specific damage. A second low dose data set is then collected and the phases obtained by finding the 'heavy' (i.e. sulfur) atom sites. The principle has been demonstrated for a protein with six disulfide bridges (bovine trypsin) and for a DNA/RNA hybrid with brominated guanine, in which radiation damage caused debromination [30••], as observed previously [31].

Although using RIP alone for structure solution may currently be confined to a limited number of favourable cases, when combined with, for example, sulfur SAD, it will undoubtedly be a very useful phasing tool.

Radiation damage has also been used, in conjunction with more conventional data collection and microspectrophotometer monitoring, to elucidate the catalytic pathway of horseradish peroxidase. The catalytic reduction of a bound dioxygen species was induced by electrons liberated during X-ray irradiation (i.e. by 'radiation damage') and was tracked using an ingenious multicrystal experimental strategy. Data sets comprising 90° in phi were collected from nine different crystals with the starting phi staggered by 10° for each. Composite 90° data sets were assembled by taking the 10° sweep from each crystal that had experienced the same X-ray dose. Electron density maps calculated from each composite data set gave 'snapshots' of the enzyme in various stages of reduction, similar to a redox titration. The radiation damage process could potentially be utilized to investigate high valency intermediates of other redox enzymes. Also, it may have unnoticeably affected the oxidation state of many redox proteins in the PDB [32••].

High and ultra-high resolution diffraction

Cryocrystallography has been a pivotal technique for the determination of the increasing number of atomic-resolution protein structures (<1.2 Å). For a given size of crystal, data quality is generally higher and the resolution limit is maximised if the mosaicity of the crystal is minimised. Thus, for high or ultra-high resolution studies, optimising the cryoprotocol to try to reproduce room temperature mosaicity is vital. The advantages of atomic resolution have been well documented [33] and the number of such structures in the PDB is increasing (161 by 2000, 236 by 2001, 303 by 2002 and 322 by June 2003), although not at the rate that the overall database is expanding. Only four ultra-high resolution (<0.8 Å) protein structures have been deposited; such high order is obviously rare in a macromolecule.

Future prospects

Having become vital for X-ray data collection, cryocrystallographic techniques must now be incorporated into high-throughput structure determination pipelines. Growing crystals in cryoprotected mother liquors will become more common, but a pivotal bottleneck for automation, yet to be overcome, is identifying a candidate crystal, cryoprotecting it, catching it in a cryoloop and then flash-cooling it. A rationally designed general strategy will be required that should include choice of cryoprotectant agent for the cryobuffer, soak temperature and length of time the crystal is in the buffer, choice of cryogen for flash-cooling and the best X-ray irradiation regime.

Radiation damage at cryotemperatures has emerged very recently as a new and important area, but experiments that vary only one parameter are hard to design and lengthy to analyse. Significant progress is being made, however, which may yet allow the flux of the undulator beamlines to be fully utilized at cryotemperatures, thus overcoming the crystallographer's current dilemma: rate of radiation damage versus diffraction intensity.

Acknowledgements

I thank Sylvie Doublie and Robin Owen for help with the *Acta D* survey, Stephen Lee for his photography, and James Murray and Martin Noble for helpful discussions.

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