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Macromolecular Crystal Annealing: Overcoming Increased Mosaicity Associated with Cryocrystallography

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Abstract

Although cryogenic data collection has become the method of choice for macromolecular crystallography, the flash-cooling step can dramatically increase the mosaicity of some crystals. Macromolecular crystal annealing significantly reduces the mosaicity of flash-cooled crystals without affecting molecular structure. The process, which cycles a flash-cooled crystal to ambient temperature and back to cryogenic temperature, is simple, quick and requires no special equipment. The annealing process has been applied to crystals of several different macromolecules grown from different precipitants and using a variety of cryoprotectants. The protocol for macromolecular crystal annealing also has been applied to restore diffraction from flash-cooled crystals that were mishandled during transfer to or from cryogenic storage. These results will be discussed in relation to crystal mosaicity and effects of radiation damage in flash-cooled crystals.

1. Introduction

The development of techniques for cryogenic data collection has been an important advance in the field of macromolecular crystallography (Garman & Schneider, 1997; Rodgers, 1997). Protection of macromolecular crystals from radiation damage at cryogenic temperatures allows collection of complete and even multiple data sets from a single crystal, and is especially important for collection of multiple wavelength anomalous dispersion (MAD) data. The only significant disadvantage of cryocrystallography is increased mosaicity in some crystals after flash-cooling. The scope of this problem has been indicated in a study of 19 crystal systems (Rodgers, 1994). In that study, only eight of the 19 crystal types exhibited little or no mosaicity increase after flash-cooling, while eight of the remaining 11 were found to exhibit a doubling of rocking curve widths (mosaicity) after flash-cooling. In most cases the advantages of cryogenic data collection far outweigh the increased mosaicity. However, increased mosaicity coupled with large unit-cell dimensions may result in overlap of Bragg reflections. A limited number of approaches are currently available to overcome flash-cooling induced mosaicity. These include a systematic survey of cryoprotectants and flash-cooling rates or the development of special-purpose software for data reduction.

This report presents a novel approach to alleviate the problem of increased mosaicity induced by flash-cooling. The process is contrary to the prevalent conception that a crystal, once flash-cooled, cannot be warmed without damage to the crystal and irreversible loss of diffraction. This study demonstrates that macromolecular crystals may be cycled easily and reproducibly between ambient and cryogenic temperatures. Surprisingly, warming of a flash-cooled crystal can reduce the effect of flash-cooling on mosaicity (Harp *et al.*, 1997) in a process that has been termed annealing because of the similarity to annealing of metals and glass. Macromolecular crystal annealing can significantly improve diffraction quality in those crystals which show increased mosaicity after flash-cooling. However, the annealing process does not improve a poorly diffracting crystal suffering from molecular disorder.

The first, serendipitous, annealing applied to a macromolecular crystal that led to a careful examination of the process is described. Critical features of the process have been identified and a standard annealing protocol developed. Finally, the results indicate that the annealing process acts at the mosaic block level of structure within the crystal and does not affect molecular structure.

2. Materials and methods

2.1. *Crystals examined*

The annealing process has been studied using the crystal systems listed in Table 1. These crystals were grown using a variety of precipitants and flash-cooled using a variety of cryoprotectants. In addition to the cryoprotectants listed, the crystals also have been flash-

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Table 1. *Summary of the crystal systems examined in this study along with growth conditions and cryoprotectants used for flash-cooling*

Macromolecule	Space group	Unit-cell parameters (Å)	Data resolution (Å)	Mass in asymmetric unit (kDa)	Crystallization agent	Cryoprotectant
Nucleosome core particle	$P2_12_12_1$	$a = 105$ $b = 110$ $c = 180$	3.1	208	$MnCl_2$	MPD/ Paratone N
Histone octamer	$P3_221$	$a = b = 117$ $c = 102$	3.0	54	NH_4SO_4	Glycerol/ Paratone N
Lysozyme	$P4_32_12$	$a = b = 78$ $c = 37$	2.0	14.6	NaCl	Glycerol + Paratone N

cooled using Paratone N to remove all surface solution from the crystal (Hope, 1988). The success of flash-cooling with Paratone N depended on the oil being moisture free. Accordingly, the oil used in this study was stored in a vacuum desiccator. Nucleosome core particles were prepared as previously described and contained a 146 bp DNA palindrome based on an alpha satellite sequence from the human X chromosome bound to the 108 kDa histone octamer (Harp *et al.*, 1996). Purified nucleosome core particles (4–8 mg ml^{-1} in 40–60 m*M* KCl, 70 m*M* $MnCl_2$, and 10 m*M* potassium cacodylate pH 6.0) were crystallized by double microdialysis to lower the Mn^{2+} concentration using the Diffusion Controlled Apparatus for Microgravity (DCAM) (Dr Daniel Carter, New Century Pharmaceuticals, Huntsville, AL, USA) and flown on the joint USA/Russian Mir 3 and Mir 5 missions. The nuceosome core particle crystals grow in space group $P2_12_12_1$ with $a = 105$, $b = 110$ and $c = 180$ Å and one nucleosome in the asymmetric unit. Crystals are prepared for data collection by dialysis into artificial mother liquor containing 22.5% 2-methyl-2,4-pentanediol (MPD) to stabilize the unit cell and improve resolution while also providing cryoprotection. Nucleosome core particle crystals grow as hexagonal rods and routinely attain 0.2–0.4 mm in diameter and up to 4 mm in length. Histone octamers were prepared as previously described (Harp *et al.*, 1996) and consist of two each of four separate subunits. Histone octamers [20 mg ml^{-1} in 2 *M* NaCl, 10 m*M* dithiothreitol (DTT), 50 m*M* sodium pyrophosphate, pH 6.5] were crystallized by microdialysis at either 277 or 294 K against 70% saturated ammonium sulfate, 10 m*M* DTT, 50 m*M* sodium pyrophosphate, pH 6.5. The crystals grow in space group $P3_221$ with unit-cell parameters of $a = b = 117$ and $c = 102$ Å and 54 kDa in the asymmetric unit. Glycerol (15%) was added as a cryoprotectant by overnight dialysis. Histone octamer crystals typically grow as 0.8 mm hexagonal bipyramids. Lysozyme (Sigma) crystals were grown by vapor diffusion using a precipitant solution of 0.6 *M* NaCl, 50 m*M* sodium acetate, pH 4.5, and a protein concentration of 20 mg ml^{-1}. Lysozyme crystals grow in space group $P4_32_12$ with unit-cell parameters of $a = b = 78$ and $c = 37$ Å and 14.6 kDa in the asymmetric unit. Crystals grew to about 1 mm and were cut in half for use. Cryoprotection was achieved by first dragging a crystal quickly through a drop containing 30% glycerol in reservoir solution and then transferring the crystal into Paratone N.

2.2. *Data collection and processing*

All data were collected using a Rigaku RU200 rotating-anode X-ray generator with a copper target, double focusing mirrors (Charles Supper Co., Natick, MA, USA) and an 18 cm Mar image-plate area detector (X-ray Research Gmbh). The cryostat consisted of a transfer tube (Molecular Structure Corp., The Woodlands, TX, USA) delivering a cold (100–90 K) nitrogen gas stream coaxial with the spindle. All crystals were mounted using rayon loops on magnetic goniometer caps (Hampton Research, Riverside, CA, USA) and were flash-cooled directly in the cold nitrogen gas stream. Data were processed using the *HKL* suite (Otwinowski, 1993), *MOSFLM* (Leslie, 1991) and the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994), and *MARXDS* (Kabsch, 1988). Similar results were obtained with all three data-reduction programs.

2.3. *Lysozyme refinement*

The 95 K tetragonal lysozyme structure, 1LSF, was positioned within the unit cell by molecular replacement using the program *AMoRe* (Navaza, 1992). The best rotation-function solution had a correlation coefficient of 17.8% using 12.0–3.0 Å data, while the translation function yielded a correlation coefficient of 65.5% and an *R* factor of 38.2%. The 1LSF coordinates, including 119 water molecules, were refined against the flash-cooled and annealed lysozyme data using the program *X-PLOR* (Brünger, 1992*a*). A random test set corresponding to 10% of the data was flagged for monitoring the free *R* factor during the course of refinement (Brünger, 1992*b*). Identical protocols, including rigid-body refinement, three cycles of positional and individual *B*-factor refinement, a 3000 K simulated annealing, and final positional and individual *B*-factor refinements were used for both data sets. The root-mean-square

(r.m.s.) deviations between the resulting structures were calculated for all atoms (Table 3) using *X-PLOR*. All of the data from 18.1 to 2.0 Å were included in the refinement using a bulk solvent correction and a 2σ cutoff.

3. Results

3.1. *Discovery of annealing in macromolecular crystals*

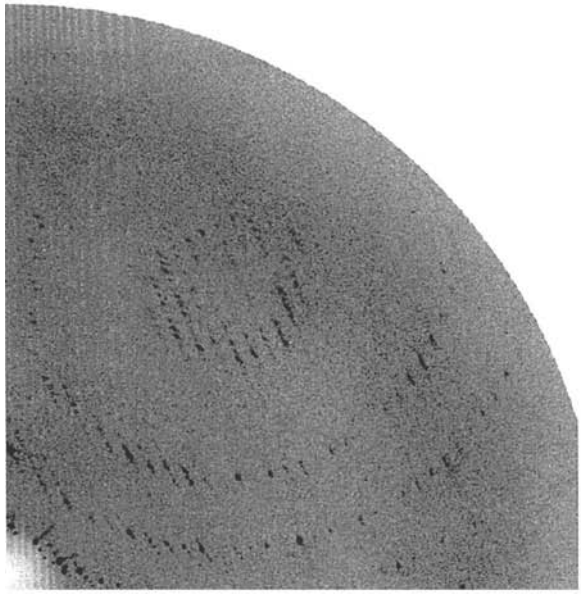
The first indication of annealing in a macromolecular crystal occurred during data collection on a nucleosome core particle crystal (Harp *et al.*, 1997). These crystals are sensitive to radiation damage at ambient temperature, making cryogenic data collection essential. Flash-cooling is possible using MPD as a cryoprotectant but the mosaicity of the crystal generally increases by a factor of 2 to 4. During a survey of cryoprotectants, a crystal was found to exhibit unacceptably high mosaicity and was removed from the cold nitrogen gas stream. It was placed in a large drop of the cryoprotectant for examination under a light microscope. The crystal remained intact and appeared more transparent than before flash-cooling. Therefore, it was flash-cooled a second time. Subsequent diffraction images showed that the quality of the crystal had dramatically improved. The diffraction images before and after annealing are compared in Fig. 1. The reduction in mosaicity of the crystal is evident by the decreased width of the lunes and the more sharply formed Bragg reflections in photographs taken over the same rotation angle.

3.2. *Essential features of the annealing process*

The protocol for annealing a crystal assumes that an adequate cryoprotectant is available or that the crystal may be flash-cooled using a hydrocarbon oil and that the crystal diffracts well. The crystal to be annealed must first be flash-cooled. The crystal is then removed from the cold nitrogen gas stream and quickly transferred to a drop of cryoprotectant or oil at the crystal growth temperature. The crystal is allowed to warm in the drop for at least 3 min. The drop should be covered to prevent evaporation. To complete the process, the crystal is remounted on a loop and flash-cooled. Two factors have been identified for successful application of the annealing protocol. The first is that the crystal must be transferred directly from the cryostat to a substantial drop of cryoprotectant solution. If the crystal was flash-cooled in Paratone, then it is transferred to a drop of Paratone for annealing. The second factor is incubation time in the cryoprotectant drop. The protocol employed in this laboratory uses a 0.3 ml drop of cryoprotectant or oil and a 3 min incubation. The incubation time for annealing can be extended, however, shortening the incubation period produced inconsistent results.

3.3. *Tool for recovery of flash-cooled crystals*

Macromolecular crystal annealing was used to recover crystals that had been mishandled during flash-cooling or cryogenic storage. Diffraction images of a mishandled crystal before and after annealing are compared in Fig. 2. In this example, a nucleosome core



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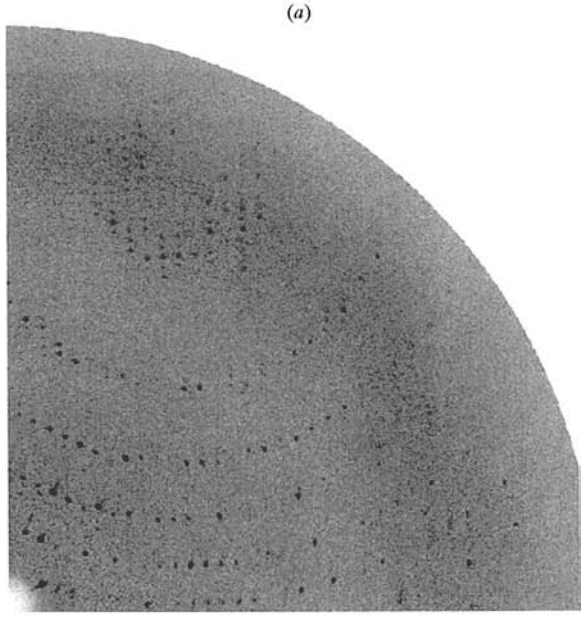

(*b*)

Fig. 1. A comparison of diffraction from the same nucleosome core particle crystal demonstrating the effect of annealing. The two wedges are taken from images recorded on a Mar image-plate area detector using the same rotation angle (0.25°) and exposure time. Image (*a*) illustrates typical diffraction after initial flash-cooling. The same crystal was then annealed, resulting in significantly improved diffraction, as shown in image (*b*).

particle crystal had been flash-cooled and X-ray diffraction data collected for over 2 d. Instrument maintenance required that the crystal be removed and stored in liquid nitrogen. Upon remounting the crystal, the diffraction (Fig. 2*a*) showed strong ice rings, high mosaicity, and low resolution not extending past 8 Å. The damaged crystal was then annealed, diffraction was restored (Fig. 2*b*) and data collection was completed. Without application of the annealing process, the crystal would have been discarded.

3.4. *Annealing highly mosaic crystals, the nucleosome core particle*

The effect of annealing on crystals of the nucleosome core particle was examined after flash-cooling and again after annealing. 20 rotation per images (0.25° rotation/image) were collected to 3.1 Å resolution over approximately 10 h for each data set. Annealing produced a greater than twofold decrease in the mosaicity of the crystal, from 0.825° after flash-cooling to 0.345° after annealing. The annealed value compares to a mosaicity of 0.275° from a similarly grown crystal measured at 277 K. Plots of linear R factors by resolution bin for each data set (Fig. 3) demonstrate that there is no significant difference other than a slight improvement in R factors after annealing, particularly for low-resolution reflections. Plots of $I/\sigma(I)$ *versus* resolution bin reveal a similar pattern (data not shown).

3.5. *Annealing moderately mosaic crystals, the histone octamer*

The effect of annealing on histone octamer crystals was examined by collecting complete data sets to 3.0 Å from each of two halves of a single crystal. One half was flash-cooled and the other was flash-cooled and then annealed. Mosaicity decreased by about 20% after annealing, from 0.338° for the flash-cooled half to 0.217° for the annealed half. The annealed value compares to a value of 0.175° measured at 277 K for a similar crystal. Plots of linear R factors by resolution bin for each data set (Fig. 4) demonstrate that there is no significant difference other than a slight improvement in R factors after annealing, particularly for low-resolution reflections. Plots of $I/\sigma(I)$ *versus* resolution bin reveal a similar pattern (data not shown). The data sets were scaled and merged to test for differences which may have been caused by the annealing process. The merging R factor of 0.031 indicates that there is no significant difference between the two data sets.

3.6. *Annealing does not affect molecular structure*

Potential effects of the annealing process on molecular structure were examined using a tetragonal lysozyme crystal. The crystal did not exhibit an increase in mosaicity after flash-cooling and annealing did not improve the quality of the crystal in this case. In other instances, lysozyme crystals have shown increased mosaicity after flash-cooling and reduction in mosaicity after annealing. Typical values of mosaicity for a lyso-

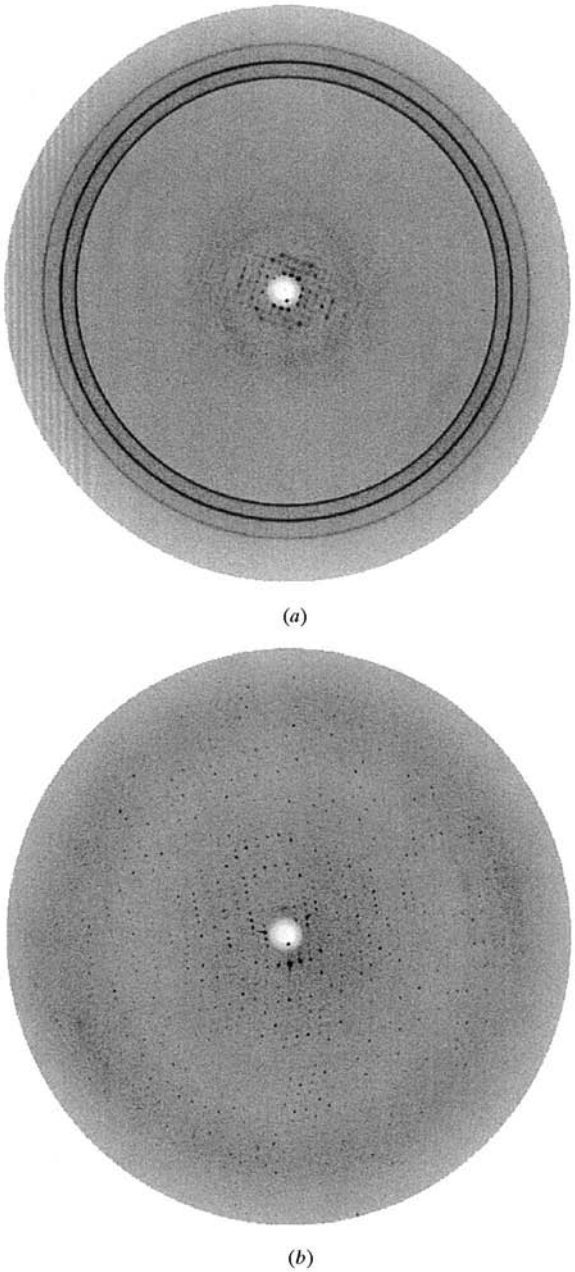

Fig. 2. An example of the use of protein crystal annealing to recover a mishandled crystal. A nucleosome core particle crystal had been flash-cooled and used to collect X-ray diffraction data for about 2 d. Instrument maintenance required that the crystal be stored in liquid nitrogen. After remounting the crystal to continue data collection, the first image (*a*) displayed signs of damage due to transient warming during handling. The crystal was annealed and diffraction returned to normal as seen in (*b*).

Table 2. *Data-collection statistics from tetragonal lysozyme*

The flash-cooled data set was taken from a crystal which was flash-cooled and the annealed data set was taken from the same crystal after three rounds of annealing and 18 h of radiation exposure prior to the final annealing. The two data sets were merged without scaling and processed as a single data set to provide the combined statistics.

Data set	Flash-cooled	Annealed	Combined
Unique reflections	8005	7992	8015
Completeness (%)	99.5	99.1	99.3
Redundancy	8.0	7.9	15.8
R_{sym} (overall)	0.043	0.043	0.045
R_{sym}† (2.16–2.02 Å)	0.086	0.085	0.089

† $R_{sym} = \sum|I - \langle I\rangle|/\sum I$.

zyme crystal at 277 K, flash-cooled, and after annealing are 0.180, 0.449 and 0.348°, respectively. Complete data sets were collected from a single flash-cooled lysozyme crystal and from the same crystal following three rounds of annealing. The flash-cooled and annealed lysozyme data are essentially identical based on the *R* factors obtained from processing the data sets separately and from processing the merged (combined) data (Table 2). Coordinates for a 95 K tetragonal lysozyme structure (1LSF) were refined against the flash-cooled and annealed data sets. The crystallographic residuals obtained during the course of the 1LSF refinement against the flash-cooled and annealed lysozyme data showed similar profiles (not shown) and converged at similar values (Table 3). The structures resulting from the two refinements showed no significant differences, with similar temperature-factor profiles (not shown) and small r.m.s. deviations between the two structures (Table 3).

4. Discussion

This report presents the discovery and first study of macromolecular crystal annealing. The reversibility of cryogenic cooling, as briefly mentioned by others (Sauer & Ceska, 1997), has been demonstrated. However, the annealing process provides significant additional benefits to cryogenic data collection. Macromolecular crystal annealing can reverse the adverse effects of increased mosaicity in flash-cooled crystals. The process can also simplify crystal handling and has the potential for recovering mishandled crystals. The annealing process has been studied using a diverse range of macromolecules which have been crystallized and flash-cooled under a variety of conditions. Lysozyme is typical of a small monomeric protein; the histone octamer is a moderate-sized multi-subunit complex; the nucleosome core particle is a large protein–DNA complex. While all three could be reversibly flash-cooled, the effects of annealing varied between the systems. The nucleosome core particle, showing the largest increase in mosaicity upon flash-cooling, also showed the largest improvements (> twofold) upon annealing. The histone octamer, showing moderate (20%) increases in mosaicity upon flash-cooling, showed moderate improvements in mosaicity. Finally, lysozyme showed the variability common to flash-cooling and, consequently, to the results of the annealing process. Some crystals showed no detrimental effects from flash-cooling and hence showed no improvements after annealing. Other lysozyme crystals showed increases in mosaicity upon flash-cooling along with moderate improvements after annealing (20%). The studies described here suggest that the annealing process is universally applicable, but improvements in mosaicity will be proportional to the adverse effects of initial flash-cooling on a particular crystal.

The annealing process also has been applied to a highly mosaic crystal of the 301 kDa ClpP protease (Flanagan *et al.*, 1997) exhibiting severe spot overlap (James Hartling, Maria Bewley & John Flanagan, personal communication). Annealing enabled a complete data set to be measured and the position of a bound inhibitor to be identified. More recently, the annealing process has been successfully applied to crystals of fumarylacetoacetate hydrolase (FAH) using 30% polyethylene glycol 400 (PEG 400) as the cryoprotectant. This result demonstrates that another class of protein crystallization agents is compatible with macromolecular crystal annealing.

The ability to reverse the flash-cooling process and to return a crystal to ambient temperature provides the potential for recovering mishandled crystals and simplifies crystal handling. The process is simple,

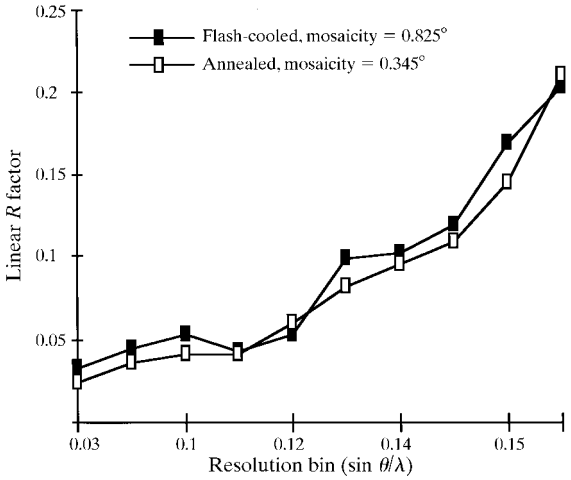

Fig. 3. The effect of annealing on diffraction from a nucleosome core particle crystal was tested by comparing 20 images (0.25° rotation per image) from the same crystal before and after annealing. The data to 3.1 Å were processed identically and the linear *R* factors plotted against resolution bins. The flash-cooled data were collected after initial flash-cooling and the annealed data were collected from the same crystal after annealing and subsequent flash-cooling.

reproducible, and requires no special equipment. The annealing process enabled a poorly diffracting nucleosome core particle crystal showing strong ice rings (see §3.1, Fig. 2) to be recovered and allowed the collection of a data set to be completed. Also, difficulties associated with controlling the orientation of flash-cooled crystals can be overcome by remounting the same crystal.

The similarities between the flash-cooled and annealed data for the three crystal systems studied indicates that annealing does not affect macromolecular structure. This conclusion is further supported by the similarity of the structures resulting from the lysozyme refinement against flash-cooled and annealed data. This study also suggests that the annealing process acts at the level of the mosaic block structure of the crystal. A mosaic crystal is defined as being composed of blocks, each of which may be a perfect crystal containing many unit cells. Mosaic spread for a Bragg reflection is produced by the angular misalignment of the blocks (see Helliwell, 1992, p. 24). Other factors contributing to refined mosaicity values (*i.e.* beam divergence, spectral bandwidth, *etc.*) were constant in the experimental setup. During the initial flash-cooling, the average misalignment of mosaic blocks can be exacerbated and the block structure may be altered along lattice stress lines or planes. Application of heat after flash-cooling, in the form of the annealing process, allows reordering of the blocks and possibly 'melts out' imperfections introduced by the initial flash-cooling. This is supported by the observation that mosaicity does not return to previous elevated levels after annealing and subsequent flash-cooling, and that additional rounds of annealing do not lead to further improvements in crystal quality.

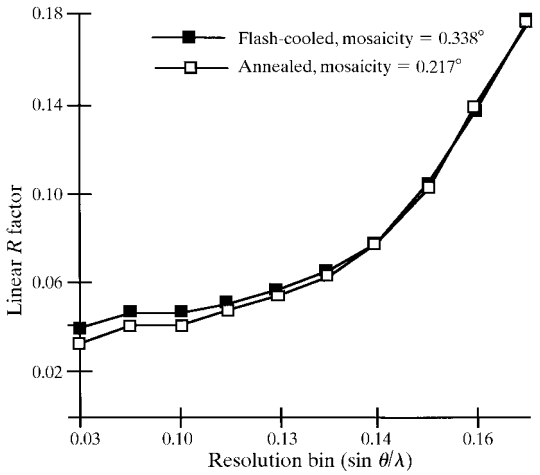

Fig. 4. Two halves of a single histone octamer crystal were used to test the effect of annealing on diffraction quality. One half of the crystal was flash-cooled and used to collect a full data set to 3.0 Å; these data were processed and the linear R factors plotted against resolution bin. The second half of the crystal was then flash-cooled, annealed and a full data set was collected (the annealed data) to compare with the flash-cooled data. The annealed data were processed identically to the flash-cooled data.

Table 3. *Refinement statistics for flash-cooled and annealed data sets taken from the same tetragonal lysozyme crystal*

The flash-cooled data were collected after initial flash-cooling and the annealed data were collected from the same crystal after three rounds of annealing and 18 h of radiation exposure prior to the final annealing. The refinements used coordinates for a 95 K lysozyme structure, 1LSF, from the Protein Data Bank.

Data set	Flash-cooled	Annealed
Initial (R_{cryst}/R_{free})†	0.3251/0.3092	0.3215/0.3242
Final (R_{cryst}/R_{free})	0.2155/0.2915	0.2107/0.3098
Mean B factors (Å^2) (m.c./s.c.)‡	17.6/19.3	16.8/19.3
R.m.s. deviations (Å) between refined models (m.c./s.c.)‡	0.19/0.29	

† $R_{cryst} = \sum \big||F_o| - |F_c|\big| / \sum |F_o|$. ‡ m.c., main-chain atoms; s.c., side-chain atoms; r.m.s., root-mean-square.

The results of macromolecular crystal annealing studies support current understanding of free radical damage under cryogenic conditions (Henderson, 1990). If it is assumed that damage to the crystal occurs through the production of free radicals and that the free radicals are trapped in the glass phase under cryogenic conditions, then returning the crystal to room temperature should liberate the free radicals with subsequent damage to the crystal lattice. Radiation damage should be apparent in diffraction from an annealed crystal, particularly for higher resolution reflections, but this was not seen in the data from the nucleosome core particle crystal (Fig. 3) subjected to 10 h exposure prior to annealing. Previous experience with data collection at 277 K has shown that 7–8 h of exposure to X-rays from a rotating-anode source resulted in about 25% reduction in intensity for high-resolution reflections (unpublished data). Further, the nucleosome core particle crystal from which the data shown in Fig. 2 were collected had previously received 2 d of exposure to Cu $K\alpha$ radiation. Annealing of the crystal involved its incubation at room temperature for several minutes during which time accumulated free radicals would be expected to react with the molecules, resulting in degraded diffraction quality. This was not evident in the subsequent data analysis. The most plausible explanation for both observations is that in each case an area of the crystal which was not previously exposed to X-rays was centered in the X-ray beam after annealing. The crystals used in the study were larger than the X-ray beam from the double focusing mirrors. Subsequent experiments have confirmed that radiation damage is apparent after annealing if care is taken to recenter the previously exposed region of the crystal.

Macromolecular crystal annealing is likely to extend the application of cryogenic data collection to more challenging crystal systems. The annealing process may

prove to be a useful research tool for the investigation of mosaic structure as well as the mechanism of radiation protection under cryogenic conditions. A particularly intriguing possible application is in the field of cryoenzymology. Physical trapping of reaction intermediates by flash-cooling could be extended to the examination of multiple reaction intermediates in the same crystal.

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