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Cryocrystallography of Influenza Virus Hemagglutinin Crystals

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Abstract

X-ray diffraction data collected at cryogenic temperatures from flash-cooled crystals of influenza virus hemagglutinin show improvements in both resolution and quality relative to data collected at 277 K. These improvements are dramatic for flash-cooled hemagglutinin crystals irradiated with X-rays from a synchrotron source. At the Cornell High Energy Synchrotron Source flash-cooled hemagglutinin crystals diffracted at least 0.9 Å farther than hemagglutinin crystals at ambient temperatures. Radiation damage in the flash-cooled crystals is reduced, making it possible to collect a complete data set from a single hemagglutinin crystal. However, radiation damage is not eliminated in the flash-cooled crystal. As a result the quality of X-ray data can be significantly degraded during long exposure times at a synchrotron source.

Introduction

The past several years have seen the development of techniques and equipment designed to facilitate the routine collection of X-ray diffraction data from crystals of biological macromolecules flash cooled to, and maintained at, cryogenic temperatures (approximately 108 K) (Petsko, 1975; Dewan & Tilton, 1987; Hope, 1988; Hope *et al.*, 1989; Teng, 1990; Young & Dewan, 1990). For conciseness and clarity the term 'flash cooled' will refer to a crystal that has been rapidly cooled to cryogenic temperatures and subsequently maintained at these temperatures during the collection of X-ray diffraction data. Crystals of biomolecules can be flash cooled without apparent external damage. Differences in the mosaic spread of flash-cooled crystals and crystals at ambient temperatures (typically 273–293 K) can be minimized with the correct choice of cryoprotectant and the slow replacement of harvest buffer with cryoprotectant (Petsko, 1975; Tilton, Dewan & Petsko, 1992). Crystal volume is typically 3–4% smaller in flash-cooled crystals relative to crystals at ambient temperatures (Hope, 1988). Unit-cell dimensions of flash-cooled crystals are reproducible thereby making it possible to generate isomorphous-difference maps from either heavy-atom soaks (Clark, Halay, Lai & Burley, 1993) or ligand soaks (Watowich, data not shown).

No consistent relationship is observed between crystal diffraction limit and temperature. Flash-cooled crystals are observed to diffract to a higher resolution than crystals at ambient temperatures (Petsko, 1975; Teng, 1990), but many protein crystals diffract similarly at cryogenic and ambient temperatures (Petsko, 1975; Hope *et al.*, 1989).

Radiation damage is virtually eliminated at cryogenic temepratures (Hope *et al.*, 1989) with some investigators observing no radiation damage to flash-cooled crystals exposed to X-rays from a Cu $K\alpha$ source (Hope, 1988). By substantially reducing radiation damage a complete data set can be collected from a single crystal. This in turn improves data quality by reducing errors associated with crystal decay and with scaling data collected from multiple crystals. The minimization of radiation damage is particularly important when crystals are radiation sensitive, are in limited supply or are exposed to intense X-ray radiation from synchrotron sources.

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The structure of bromelian-released influenza A hemagglutinin (BHA) was initially determined to approximately 3 Å resolution (Wilson, Skehel & Wiley, 1981). Since that time the structures of several single-site mutant hemagglutinins (Knossow, Daniels, Douglas, Skehel & Wiley, 1984; Weis *et al.*, 1990), and of hemagglutinin complexed with cellular receptor analogs (Weis *et al.*, 1988; Sauter *et al.*, 1992) have been described. These data sets are of similar quality. The resolution limit of these data sets is approximately 3 Å and the cumulative R_{sym} is approximately 10%, where $R_{sym} = (\sum_{hkl}\sum_{obs}|I^{hkl}_{obs} - \langle I^{hkl}\rangle|)/(\sum_{hkl}\sum_{obs} I^{hkl}_{obs})$. The quality and resolution limit of diffraction data from BHA crystals at ambient temperatures are independent of the X-ray radiation source since collection on either a Cu $K\alpha$ rotating anode, at the Cornell High Energy Synchrotron Source (CHESS) or at the Stanford Synchrotron Radiation Laboratory (SSRL) gave similar results (Weis *et al.*, 1988). Hemagglutinin crystals are radiation sensitive, and show a decrease in the intensity of medium-resolution diffraction data after 12–24 h of irradiation from a Cu $K\alpha$ source. At least ten large crystals (greater than 0.7 × 0.7 × 0.6 mm) are needed to complete each 3 Å data set.

This last factor makes BHA crystals a promising candidate for cryocrystallographic experiments. As discussed below, flash-cooled BHA crystals have significantly extended lifetimes when irradiated; a complete high-resolution data set can be collected from a single crystal. In addition, flash-cooled BHA crystals irradiated with X-rays from a synchrotron source diffract at least 0.9 Å farther than BHA crystals at ambient temperatures.

Experimental

Hemagglutinin crystals were grown in sitting drops. The protein concentration ranged from 40 to 80 mg ml^{-1} in 150 m*M* sodium chloride. Wells contained 1.38–1.42 *M* sodium citrate (pH 7.5) and 0.1%(*w*/*v*) sodium azide. The conditions are similar to those described earlier (Wilson *et al.*, 1981). Crystals grew after 1–3 months and were typically 0.7 mm on an edge when harvested into a buffer of 1.41 *M* sodium citrate (pH 7.5) and 0.1% sodium azide.

Crystals used at ambient temperatures were mounted in glass capillaries and cooled to 277 K for data collection. Prior to flash cooling, BHA crystals were transferred through solutions containing harvest buffer, increasing concentrations of ligand (for the cases where data was collected from BHA–ligand complexes) and the cryoprotectant xylitol (Fluka BioChemika, Switzerland). The crystals were initially transferred from harvest buffer to a solution of harvest buffer, ligand (when appropriate) and 94 m*M* xylitol. Four subsequent transfers occurred with the concentration of ligand (when appropriate) and cryoprotectant doubled at each transfer. The final solution into which the BHA crystals were transferred contained harvest buffer, ligand (when appropriate) and 1.5 *M* xylitol. Crystals from this final solution were supported in 80 μm wire Nichrome loops (Teng, 1990) and flash cooled in a stream of dry nitrogen gas at 113 K (Hope, 1988).

X-ray diffraction data were collected either in our laboratory or at the Cornell High Energy Synchrotron Source. Collection parameters are given in Table 1. In our laboratory Cu $K\alpha$ X-rays were generated by an Enraf–Nonius GX-13 rotating anode operating at 2.4 kW (40 kV, 60 mA) and fitted with a 100 μm focus cup, Franks double-focus mirrors (Harrison, 1968) and a 150 μm pinhole. Diffraction data were recorded with a Xentronics area detector (Durbin *et al.*, 1986). Reflections were indexed and integrated with *XDS* (Kabsch, 1988*a*,*b*) then scaled and averaged using the *CCP*4 programs *ROTAVATA*/*AGROVATA* (SERC Daresbury Laboratory, 1979).

At CHESS all diffraction experiments were performed at the F1 beamline station (λ = 0.905 Å). A 200 μm collimator was used when collecting native BHA data. A 300 μm collimator was used when collecting data from the BHA–ligand complexes. Diffraction data were recorded on Fuji imaging-phosphor plates (25 × 20 cm). Crystals were initially flash cooled in our laboratory, stored in a dewar containing liquid nitrogen and transported to CHESS where they were remounted in a nitrogen stream at cryogenic temperatures. The cryogenic cooling device for the CHESS F1 beamline was kindly provided by A. Yonath and colleagues and is similar to the device shown in Fig. 3(*a*) of Hope *et al.* (1989). Crystals treated in this manner diffracted similarly to crystals flash cooled at CHESS (data not shown). Crystals of native BHA, BHA soaked with 50 μ*M* 2-*O*-6′-(naphthylmethyleneamidehexyl)-4-*O*-glycyl-dansyl-sialic acid and BHA soaked with 1 m*M* 2-*O*-6′-(naphthylmethyleneamidehexyl)-sialic acid were positioned in the cryogenic stream for 33, 21 and 19 h, respectively. Total irradiation times were 5.3, 6.3 and 3.5 h, respectively. A 15 h delay was experienced between the time the native BHA crystal was mounted at CHESS and the time data collection began; during this interval the crystal remained positioned in the cryogenic stream with no apparent damage. Frames were indexed and integrated with *DENZO* (Otinowski, 1985) Scaling, averaging and post refinement of the recorded reflections was performed with either the *CCP*4 programs *ROTAVATA*/*AGROVATA*/*POSTREF* (SERC Daresbury Laboratory, 1979) or with *SCALEPACK* (Otinowski, 1985).

Table 1. *Characterization of crystallographic data sets*

	BHA + NeuAc2N4	BHA + NeuAc4D	BHA	BHA + NeuAc2N6	BHA + NeuAc4D2N6
Temperature (K)	277 ± 2	113 ± 1	108 ± 2	108 ± 2	108 ± 2
X-ray source	Rotating anode, double-focusing mirrors, 150 μm pinhole, λ = 1.54 Å	Rotating anode, double-focusing mirrors, 150 μm pinhole, λ = 1.54 Å	Synchrotron (CHESS F1 beamline), 200 μm collimator, λ = 0.905 Å	Synchrotron (CHESS F1 beamline), 300 μm collimator, λ = 0.905 Å	Synchrotron (CHESS F1 beamline), 300 μm collimator, λ = 0.905 Å
Detector	Area detector	Area detector	Imaging-phosphor plate	Imaging-phosphor plate	Imaging-phosphor plate
Frame size (°)	0.04	0.04	0.75	0.6	0.6
Exposure time/degree (min)	150	125	4 (φ = 0–40.5°) 2 (φ = 41.25–120°)	1.39 (φ = 0–44.4°) 3.33 (φ = 45–90°)	4.17
Crystal size (mm)	11 crystals (~0.7 × 0.7 × 0.6)	0.6 × 0.6 × 0.5	1.0 × 0.8 × 0.8	1.0 × 0.8 × 0.8	0.6 × 0.6 × 0.6
Data-reduction software	*XDS*, *ROTAVATA*, *AGROVATA*	*XDS*, *ROTAVATA*, *AGROVATA*	*DENZO*, *ROTAVATA*, *AGROVATA*, *POSTREF*	*DENZO*, *SCALEPAK*	*DENZO*, *ROTAVATA*, *AGROVATA*, *POSTREF*
Unit cell (Å)	$P4_1$ a = 163.6, c = 177.8	$P4_1$ a = 160.6, c = 175.4	$P4_1$ a = 160.4, c = 175.4	$P4_1$ a = 160.8, c = 175.7	$P4_1$ a = 160.3, c = 175.2
Resolution (Å)	3.0	2.6	2.2	2.1	2.1
R_{sym} (%)	8.0 (12–3.0 Å)	7.2 (12–2.6 Å) 6.5 (12–3.0 Å)	7.2 (12–2.2 Å) 6.1 (12–3.0 Å)	7.0 (12–2.1 Å) 6.8 (12–3.0 Å)	5.3 (12–2.1 Å) 4.1 (12–3.0 Å)
Completeness (%)	85 (12–3.0 Å)	71 (12–2.6 Å)	85 (12–2.2 Å)	82 (12–2.1 Å)	79 (12–2.1 Å)

Abbreviations: BHA, bromelian-released hemagglutinin; NeuAc2N4, α-2-*O*-4′-(naphthylmethyleneamidebutyl)-sialic acid; NeuAc4D, α-2-*O*-methyl-4-*O*-dansylglycyl-sialic acid; NeuAc2N6, α-2-*O*-6′-(naphthylmethyleneamidehexyl)-sialic acid; NeuAc4D2N6, α-2-*O*-6′-(naphthylmethyleneamidehexyl)-4-*O*-dansylglycyl-sialic acid.

Results

BHA crystals at 277 K

Hemagglutinin crystals at 277 K diffract weakly to approximately 3 Å resolution when irradiated with X-rays from a rotating-anode source. Approximately 2.5 h of irradiation were required to collect diffraction data from each degree of crystal rotation about the oscillation axis. Reflections from each degree of crystal rotation were grouped into data batches. Batches were scaled to one another to maximize the agreement between equivalent reflections in different batches (Fox & Holmes, 1966). The intensities of the reflections in each batch were modified such that $I_{scaled} = k_i I_{obs} \exp[(-B_i \sin^2\theta/\lambda^2]$ where k_i and B_i are constants for the ith batch.

The parameter B associated with each batch provides a measure of the strength of the high-angle reflections. Large negative values indicate the high-resolution reflections are weak relative to a reference data set. The parameter B for data collected from BHA crystals at 277 K decreased monotonically between 0 and 15 h of irradiation, implying that the intensity of high-angle reflections became progressively weaker (Fig. 1). After 15 h irradiation the crystal was translated to expose a fresh volume of the crystal to the X-ray beam. At this point the parameter B returned to the value observed when the crystal was initially irradiated. However, as this volume of the crystal was further irradiated the parameter B again decreased, indicating that the intensities of the high-angle reflections were again attenuated. This attenuation may have been the result of crystal absorption, non-isotropic crystal diffraction or radiation damage, since for a single crystal the irradiation time was coupled to rotation about the oscillation axis. However, it is likely that the first two effects had only minor influence on the observed attenuation since plots of B as a function of irradiation time gave similar curves for the 11 BHA crystals that contributed to a complete data set. Since the different crystals at 277 K were mounted in arbitrary orientations no correlation existed between an absolute crystal orientation and the irradiation

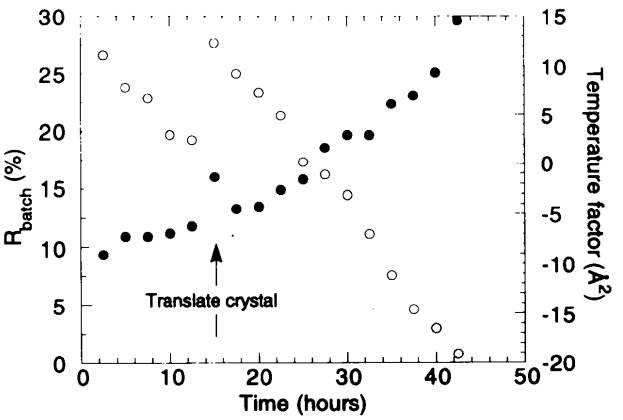

Fig. 1. Plot of R_{batch} (filled circles) and B (open circles) *versus* irradiation time for diffraction data to 3 Å resolution collected from a single BHA crystal soaked with α-2-*O*-4′-(naphthylmethyleneamidebutyl)-sialic acid and cooled to 277 K. X-ray source was a rotating anode. Additional collection parameters are given in Table 1. Each point contains reflections from a 1° rotation of the crystal about the spindle axis. The crystal was translated after 15 h of irradiation so that a new volume of the crystal was exposed to the X-ray beam.

time. Thus, the attenuation of the high-angle reflections observed as a function of irradiation time is likely to result from radiation damage.

The quality of the reflections in each batch were monitored by an R_{batch} statistic which is the R_{sym} calculated for an individual batch. This quantity can be expressed as $R_{batch} = \sum_{hkl}\sum_{batch\,i}|I^{hkl}_{batch\,i} - \langle I^{hkl}_{obs}\rangle|/\sum_{hkl}\sum_{batch\,i} I^{hkl}_{batch\,i}$ and provides a criterion to determine the agreement between reflections in a given batch with equivalent reflections in the data set as a whole. As shown in Fig. 1 the data quality from BHA crystals at 277 K was degraded as a function of irradiation time. Reflections collected after 20 h of irradiation (8° of crystal rotation) merged poorly, particularly in the high-resolution shells, with the entire data set collected from this crystal. Data collected after 20 h irradiation were not included in the final merged data set.

Flash-cooled BHA crystals

When irradiated with X-rays from a rotating-anode source, flash-cooled BHA crystals diffracted 0.4 Å farther than BHA crystals at 277 K (Table 1). The unit-cell volume decreased 4.9% in the flash-cooled crystals relative to crystals at 277 K.

Reflections from flash-cooled crystals had full-width profiles between 0.15 and 0.20°. The values are similar to the 0.14° full-width profiles measured for reflections from crystals at 277 K. The full-width profile of reflections from flash-cooled crystals and crystals at 277 K were uniform and isotropic; no correlation was observed between crystal oritentation and the full-width profile of the recorded reflections. The full-width profile of the reflections remained constant during data collection (data not shown).

A single flash-cooled BHA crystal was continuously irradiated over a 12 d period with X-rays from a rotating-anode source. No apparent degradation in data quality was observed (Fig. 2). The parameters R_{batch} and B oscillated smoothly over a narrow range during the collection period. In this case the collection time correlated with crystal orientation and the small variations observed in R_{batch} and B are likely to have resulted from absorption effects. Radiation damage appeared to be eliminated in the flash-cooled crystal since the R_{batch} values calculated for the latter third of the data collection (173–275 h) were lower than the R_{batch} values calculated during the first 75 h of data collection.

Since no radiation damage was observed, a complete data set could be collected from a single BHA crystal. The cumulative R_{sym} to 3 Å resolution was approximately 2% lower for the data set collected from a flash-cooled BHA crystal than for the best data set obtained from BHA crystals at 277 K. All data sets collected in our laboratory from flash-cooled BHA crystals were of similar quality.

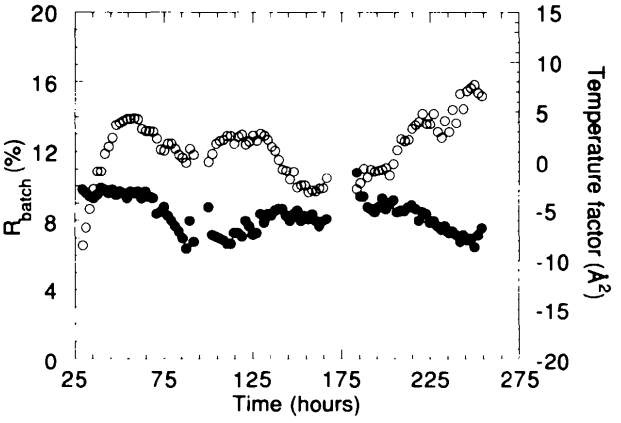

Fig. 2. Plot of R_{batch} (filled circles) and B (open circles) *versus* irradiation time for diffraction data to 2.6 Å resolution collected from a flash-cooled BHA crystal soaked with α-2-*O*-methyl-4-*O*-dansylglycyl-sialic acid. X-ray source was a rotating anode. Additional collection parameters are given in Table 1. Each point contains reflections from a 1° rotation of the crystal about the spindle axis. A complete data set to 5 Å resolution was collected during the initial 25 h of data collection (data not shown).

Synchrotron-irradiated flash-cooled crystals.

Flash-cooled BHA crystals diffract to approximately 2.1 Å resolution when irradiated with X-rays generated at the CHESS F1 beamline ($\lambda = 0.905$ Å). Collecting the weak high-angle reflections required long exposure times that were limited by the low-angle reflections saturating the Fuji imaging-phosphor plate (effective dynamic range of 10^4 when used with the Fuji scanning system).

As shown in Fig. 3, the data quality from synchrotron-irradiated flash-cooled crystals, as monitored by R_{batch} statistics, remained approximately 5% for the first 3.5 h ($\varphi = 50°$). After this time R_{batch} increased monotonically such that at 6 h irradiation the R_{batch} was approximately 19%. Reflections collected after 4.2 h merged poorly with reflections collected earlier and were not included in the final data set.

The general trend of the parameter B was downward (Fig. 3), indicating the intensities of high-angle reflections were attenuated upon increased exposure to the X-ray beam. These observations are representative of all three data sets collected at CHESS from flash-cooled BHA crystals. The behavior of B and R_{batch} shown in Fig. 3 was similar to that observed for data sets collected from radiation-damaged BHA crystals at 277 K and was in contrast to the small variations of B and R_{batch} observed in data collected from flash-cooled crystals irradiated by a rotating-anode source. Thus, it is likely that radiation damage occurred in the synchrotron-irradiated flash-cooled crystals.

The radiation-induced decrease in data quality was accompanied by an increase in the full-width profile of the recorded reflections measured from three different crystals mounted in arbitrary orientations. Fig. 4 plots the full-width of reflections (calculated during post refinement) recorded at CHESS from three different flash-cooled crystals. The full width of the reflections from these crystals increased from approximately 0.2 to 0.5° during data collection. An exception was observed for flash-cooled BHA crystals soaked with the ligand α-2-*O*-6′-(naphthylmethyleneamidehexyl)-sialic acid. The full-width profile of reflections from this crystal was intially 0.4° and increased twofold over the course of the data collection.

An additional correlation was observed between the increase in the full width of recorded reflections and exposure time per frame (Fig. 4). The full width of reflections from a flash-cooled crystal of BHA soaked with α-2-*O*-6′-(naphthylmethyleneamidehexyl)-sialic acid (open circles, Fig. 4) was constant during the first hour of irradiation and then increased rapidly from 0.4 to 0.8°. This change occurred at the point where the exposure time was increased from 50 s per frame to 2 min per frame. A less dramatic change in the slope of the curve of full width of reflections *versus* exposure time was observed for the flash-cooled BHA crystal (filled circles, Fig. 4). After 2.8 h total irradiation the exposure time was decreased from 180 s per frame to 90 s per frame. At this point the slope of the curve of full width of reflections *versus* exposure time decreased, indicating the rate of radiation damage was less severe for frames collected with the shorter frame exposure time. Thus, it appears that a component of the radiation-induced damage observed during long exposures is related to the time an individual frame is continuously irradiated.

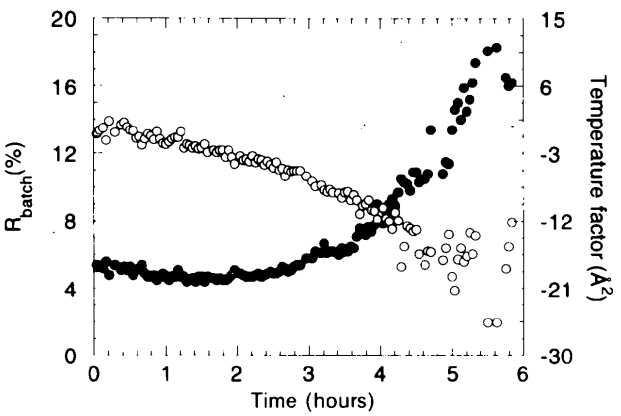

Fig. 3. Plot of R_{batch} (filled circles) and *B* (open circles) *versus* irradiation time for diffraction data to 2.1 Å resolution collected from a flash-cooled BHA crystal soaked with α-2-*O*-6′-(naphthylmethyleneamidehexyl)-4-*O*-dansylglycyl-sialic acid. X-rays were generated at the F1 beamline at CHESS. Additional collection parameters are given in Table 1. Each data point contains reflections from a 0.6° oscillation of the crystal about the spindle axis.

Discussion

When irradiated with X-rays generated by a rotating-anode, flash-cooled hemagglutinin crystals were observed to have substantially longer lifetimes relative to hemagglutinin crystals at 277 K. A complete data set was collected from a single flash-cooled hemagglutinin crystal over the course of 14 d of continuous irradiation. No radiation damage was observed during this collection period. In addition the quality and resolution of the diffraction data from flash-cooled crystals improved relative to data collected from crystals at 277 K.

When irradiated with a synchrotron source dramatic improvements were observed in the quality and resolution of the diffraction data from flash-cooled crystals relative to crystals at 277 K. In this case the flash-cooled crystals diffracted approximately 0.9 Å farther than crystals at 277 K. A complete data set to 2.1 Å resolution could be collected from a single flash-cooled BHA crystal. It is possible that not all of the improvement observed in the diffraction data is attributable to flash cooling since crystals at the

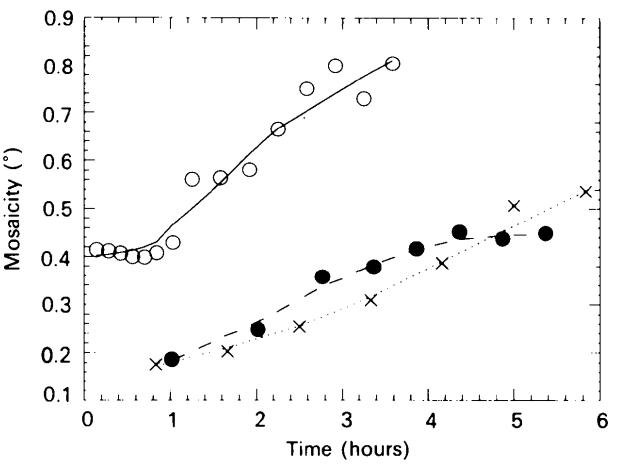

Fig. 4. Plot of the reflection full-width profile *versus* irradiation time for three high-resolution diffraction data sets collected from flash-cooled BHA crystals. Data was collected at the CHESS F1 beamline. Curves are for a BHA crystal (filled circles), a BHA crystal soaked with α-2-*O*-6′-(naphthylmethyleneamidehexyl)-sialic acid (open circles) and a BHA crystal soaked with α-2-*O*-6′-(naphthylmethyleneamidehexyl)-4-*O*-dansylglycyl-sialic acid (crossed lines). Each point in the BHA curve represents the full-width profile from 15° of rotation of the crystal about the spindle axis. Points in the curves of the BHA–ligand complexes represent the full-width profile from 6° of rotation of the crystal about the spindle axis.

synchrotron were irradiated with shorter wavelength X-rays than crystals in the laboratory.

Substantial radiation damage was observed in the flash-cooled crystals when continuously irradiated with synchrotron generated X-rays for long periods of time. These relatively long exposure times were necessary to collect weak high-angle reflections. However, to collect a good quality high-resolution complete data set from flash-cooled BHA crystals the exposure times must be balanced by the requirement that radiation damage be minimized. To this end several strightforward approaches are possible. Total irradiation time can be limited (before the R_{batch} degrades) and the exposure time per frame adjusted to ensure a complete data set is collected during this time. Alternatively long frame exposure times can be used and the irradiated crystal replaced with a fresh crystal once radiation damage is observed. Data from several flash-cooled crystals would then be required to yield a complete data set. With this approach a high-resolution data set was collected from ten flash-cooled crystals of reverse transcriptase (D. Rodgers *et al.*, personal communication). Finally, since the full-width profile of the reflections appears to be influenced by the frame exposure time, it is possible that the observed radiation damage is partly the result of heating the crystal with the synchrotron-generated beam. Thus, irradiating a flash-cooled BHA crystal for several short periods per frame rather than a single long exposure may reduce radiation damage.

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