Reduction of lattice disorder in protein crystals by high-pressure cryocooling

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Received 10 November 2015

Accepted 2 December 2015

Edited by J. Hajdu, Uppsala University, Sweden

Keywords: high-pressure cryocooling; protein crystal disorder; mosaicity; nonmerohedral twinning.

High-pressure cryocooling (HPC) has been developed as a technique for reducing the damage that frequently occurs when macromolecular crystals are cryocooled at ambient pressure. Crystals are typically pressurized at around 200 MPa and then cooled to liquid nitrogen temperature under pressure; this process reduces the need for penetrating cryoprotectants, as well as the damage due to cryocooling, but does not improve the diffraction quality of the as-grown crystals. Here it is reported that HPC using a pressure above 300 MPa can reduce lattice disorder, in the form of high mosaicity and/or nonmerohedral twinning, in crystals of three different proteins, namely human glutaminase C, the GTP pyrophosphokinase YjbM and the uncharacterized protein lpg1496. Pressure lower than 250 MPa does not induce this transformation, even with a prolonged pressurization time. These results indicate that HPC at elevated pressures can be a useful tool for improving crystal packing and hence the quality of the diffraction data collected from pressurized crystals.

1. Introduction

Crystals of macromolecules are never perfect, owing to the weakness and non-specificity of the interactions between the molecules. The best that can be hoped for is an 'ideally imperfect' crystal in which the unit cells are the same throughout the crystal and the misorientation of 'mosaic blocks' is very small (Helliwell, 1988). In real crystals, a number of kinds of disorder are observed which degrade the uniformity of the molecular packing, resulting in a decrease in the quality of diffraction data collected from these crystals (McPherson, 1999; Helliwell, 2008); structure determination becomes more difficult – sometimes impossible – and the structures obtained are less interpretable. In spite of extensive work in optimization of crystallization conditions, poorly diffracting crystals are common. Clearly, any means of reducing lattice disorder in crystals has significant value for structural biology.

Probably the most common disorder is high mosaicity. Mosaicity may be modeled as the misorientation of small 'blocks', or crystallites, making up the crystal (Nave, 1998) (see Fig. 1a). The mosaic spread of a good crystal at room temperature is typically $<0.1^\circ$ (Dobrianov *et al.*, 1998) and increases to $0.2\text{--}1.5^\circ$ in a crystal cryocooled to $\sim 100\text{ K}$, as is commonly done to extend the lifetime of crystals in the X-ray beam (Mitchell & Garman, 1994; Nave, 1998). However, crystals of molecules that do not have a single well defined lattice packing can exhibit much higher mosaic spreads. Data reduction programs [e.g. *HKL-2000* (Otwinowski & Minor,

Figure 1

Models of mosaicity and nonmerohedral twinning. (a) A mosaic crystal is made up of small blocks of varying orientation. As shown in this paper, application of pressure (arrow) can reduce the misorientation of the blocks. In a nonmerohedrally twinned crystal, the separate crystal domains align in fewer than three dimensions and the diffraction pattern contains interpenetrating lattices. (b) Nonmerohedral twinning with a monoclinic unit cell; the blue and yellow domains have different orientations of the cell (upper left cartoon) and produce the blue and yellow sets of spots in the diffraction pattern (lower left cartoon). Green spots contain contributions from both lattices. Under pressure (arrow), the monoclinic twin can convert to a single orthorhombic crystal (right). (c) Similar to (b), but with twinning in which two cell dimensions are nearly identical; blue and yellow domains have cells rotated by 90° relative to each other. Although not apparent here, the cells could be monoclinic with β near 90° rather than true orthorhombic. This is almost pseudomerohedral twinning. Again, pressure can convert the twin to a single crystal.

1997), *mosflm* (Leslie & Powell, 2007), *XDS* (Kabsch, 2010)] are very capable of processing data with mosaic spreads of up to a few degrees, provided that the data collection regime has been able to produce non-overlapped spots on images. However, as mosaicity increases avoiding spot overlap becomes increasingly difficult, and the resolution limit of useful diffraction worsens.

Another common disorder is twinning, which occurs in various forms; all involve the presence of domains containing the same unit cell, but in different orientations (Yeates & Fam, 1999). Merohedral twinning can occur in tetragonal, trigonal, hexagonal and cubic symmetries, where the equality of two (or three) lattice dimensions allows non-equivalent orientations of unit cells to produce exactly superimposable reciprocal lattices. Diffraction patterns appear normal, but twinning can be identified by statistical tests on integrated intensities, which contain contributions from two or more distinct lattices (Yeates, 1997; Parsons, 2003).

Pseudomerohedral twinning is similar to merohedral, but involves cell dimensions which coincidentally appear to have higher symmetry than is actually present, e.g. an orthorhombic cell with two equal dimensions looks like tetragonal, or a monoclinic cell with $\beta = 90^\circ$ looks like orthorhombic. In this case also, diffraction patterns appear normal while intensity distributions do not, except that when the cell dimensions do not exactly fit a higher symmetry some splitting or streaking of spots may be observed (Dauter, 2003). In nonmerohedral twinning, two reciprocal cell axes align in all domains, while the third does not. The result is diffraction patterns in which some spots contain contributions from both domains, while others are only partially overlapped or fully separated (Dauter, 2003). Figs. 1(b) and 1(c) show cartoons of nonmerohedral and (almost) pseudomerohedral twinning, respectively.

Software has been developed to process merohedrally or pseudomerohedrally twinned macromolecular crystals (Brünger *et al.*, 1998; Sheldrick, 2010; Adams *et al.*, 2010), making it possible to determine crystal structures using the diffraction data collected on such crystals (Borshchevskiy *et al.*, 2010; Dauter, 2003; Parsons, 2003). For nonmerohedrally twinned macromolecular crystals, the issue is more complicated. If an indexing routine can identify one major lattice, the data can be collected and interpreted as usual, ignoring the presence of the second, minor, lattice and accepting that some measured intensities will be inaccurate (Yeates, 1997). A recent development in *mosflm* (Powell *et al.*, 2013) allows indexing of multiple lattices in some cases, but the difficulty of integrating overlapped reflections remains. A procedure for separating partially overlapped reflections (Duisenberg *et al.*, 2003) can help but is not widely used. If an indexing routine cannot identify one, or a few, major lattice(s), the only option is to search for new crystallization conditions giving untwinned crystals.

High-pressure cryocooling (HPC), first carried out during the initial development of low-temperature protein crystallography (Thomanek *et al.*, 1973) but subsequently neglected owing to the development of cryoprotectants, has been

developed in our laboratory over the past several years as an alternative to standard cryocooling (Kim *et al.*, 2005). For this technique, crystals (or other samples) are pressurized with helium gas, typically to 200 MPa, and cooled to liquid nitrogen temperature under pressure. The pressure is then released and no special handling is required thereafter. As long as the temperature remains below 100 K, the effects of pressure cooling are 'frozen in'. By using HPC, damage due to cryocooling may be significantly reduced, and penetrating cryoprotectants are often not required. Data obtained from pressure-cryocooled crystals can be of very high quality (Kim *et al.*, 2006), and high-resolution structures have been obtained using HPC (Albright *et al.*, 2006; Kang *et al.*, 2014). The protective effect of pressure during cryocooling appears to result from the conversion of the water in macromolecular crystals from low-density amorphous (LDA) to high-density amorphous (HDA) ice (Kim *et al.*, 2008, 2009, 2015).

However, pressure, a thermodynamic variable like temperature, affects everything in the crystal, not just the water. For example, the protein citrin changes its color as a function of pressure, owing to small shifts in the position of a bound chromophore (Barstow *et al.*, 2009). Pressurization to 330 MPa in a diamond anvil cell improves the order of crystals of the virus CpMV (Girard *et al.*, 2005). HPC can sometimes alter crystal packing, for example changing the space group of FAE (the feruloyl esterase module of xylanase 10B from *Clostridium thermocellum*) from $P2_12_12_1$ to $P4_12_12$ (van der Linden *et al.*, 2014). With pressurization to 200 MPa, the technique has not been observed to improve diffraction quality over that in the as-grown room-temperature crystals. We now report cases in which the use of higher pressure, over 300 MPa, has reduced disorder due to high mosaicity and/or converted nonmerohedrally twinned crystals to single crystals.

2. Material and methods

2.1. Expression, purification and crystallization

The cDNA encoding human glutaminase C (GAC, residues 71–598) was PCR (polymerase chain reaction) amplified from the plasmid GC-T0320 (GeneCopoeia) and inserted into the expression vector pQE80. The recombinant plasmid pQE80-GAC was transferred into *Escherichia coli* BL21(DE3) for protein expression, which was induced at 291 K for 20 h with 0.3 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG). The protein was purified by fast protein liquid chromatography (FPLC) using a nickel-affinity column followed by a Superdex200 sizing column. The fractions containing GAC were pooled, concentrated and stored at 193 K for subsequent use. Native crystals were grown by the hanging drop vapor diffusion method at 293 K. Typically, 2 μ l of protein stock (20 mg ml⁻¹, in 150 mM NaCl, 5 mM Tris-HCl pH 7.5) was mixed with 2 μ l of the reservoir solution [12% PEG 6000 (*w/v*), 1.0 M LiCl, 1% dimethyl sulfoxide (DMSO) (*v/v*), 0.1 M Tris-HCl pH 8.5]. Crystals were observed after 24 h and reached a typical size of 200 $\times$ 200 $\times$ 100 μ m one week later. To make the GAC-inhibitor complex, 18 μ l of GAC solution

(20 mg ml⁻¹) was mixed with 2 μ l of inhibitor solution (20 mM, in DMSO) and incubated on ice for one hour. The mixture was centrifuged at 16 000g for 3 min and the supernatant was used for crystallization, using the same conditions as for the native protein.

The cDNA encoding the GTP pyrophosphokinase YjbM was PCR amplified from the genomic DNA of *Bacillus subtilis* and inserted into the expression vector pQE80. The recombinant plasmid pQE80-YjbM was transferred into *E. coli* BL21(DE3) for protein expression, which was induced at 219 K for 20 h with 0.3 mM IPTG. The protein was purified by FPLC using a nickel-affinity column followed by a Superdex200 sizing column. Crystals were grown by the hanging drop vapor diffusion method at 293 K. Typically, 2 μ l of protein stock (10 mg ml⁻¹, in 150 mM NaCl, 5 mM Tris-HCl pH 7.5) was mixed with 2 μ l of the reservoir solution [10% PEG 400 (*v/v*), 0.2 M CaCl₂, 0.1 M Mes-HCl pH 6.0]. Crystals reached a typical size of 30 $\times$ 30 $\times$ 500 μ m one week later.

The cDNA encoding the C-terminal domain (residues 298–598) of lpg1496 was PCR amplified from the genomic DNA of *Legionella pneumophila* and inserted into the expression vector pET28-His-SUMO. The recombinant plasmid pET28-His-SUMO-lpg1496 was transferred into *E. coli* BL21(DE3) for protein expression, which was induced at 291 K for 20 h with 0.3 mM IPTG. The His-SUMO-tag was cleaved off and the protein was purified by FPLC using a nickel-affinity column followed by a Superdex200 sizing column. Crystals were grown by the hanging drop vapor diffusion method at 293 K. Typically, 2 μ l of protein stock (10 mg ml⁻¹, in 20 mM NaCl, 20 mM Tris-HCl pH 7.5) was mixed with 2 μ l of the reservoir solution [10% PEG 4000 (*w/v*), 0.1 M Mes-HCl pH 6.5]. Crystals reached a typical size of 10 $\times$ 100 $\times$ 200 μ m two weeks later. To express Se-labeled protein, BL21(DE3) cells containing recombinant plasmid pSUMO-lpg1496 were cultured in 50 ml of lysogeny broth at 310 K overnight. The cells were pelleted by centrifugation and washed twice with 50 ml of autoclaved 0.15 M NaCl. The cells were transferred to 1 l of M9 medium containing 0.4% glucose and cultured at 310 K for 8 h. Then amino acids (Lys, Phe, Thr 100 mg l⁻¹; Ile, Leu, Val 50 mg l⁻¹) and L-Se-Met were added to the culture. After shaking at 310 K for 15 min, the culture was cooled to 291 K and 0.1 mM IPTG was added to induce protein expression. The Se-labeled protein was purified and crystallized according to the same protocol as for native protein.

2.2. High-pressure cryocooling

The oil-coating hydration method was performed as described by Kim *et al.* (2005) with modifications. A large excess of NVH oil (Hampton Research, Aliso Viejo, CA, USA) was directly applied to cover the mother droplet containing the desired crystals on a cover slide. Crystals were gently pushed from the mother droplet into the oil with a cat whisker. The crystals were picked up in a cryoloop (Hampton Research) with a minimal droplet of oil, gently touching the cover slide several times to remove excess oil. For each type of

Table 1

Diffraction data collection and processing statistics.

APC: ambient-pressure cryocooling; HPC: high-pressure cryocooling; N/D: not determined. Values in parentheses are for the highest-resolution shell.

Protein	GAC			GAC-inhib#18		YjbM		lpg1496		Se-lpg1496	
	APC†	APC‡	HPC	APC	HPC	APC	HPC	APC	HPC	APC	HPC
Pressure (MPa)	0.1	0.1	350	0.1	350	0.1	350	0.1	350	0.1	350
Space group	N/D	$P22_12_1$	$P22_12_1$	$P22_12_1$	$P22_12_1$	$P2_1$	$P2_1$	N/D	$P4_22_12$	N/D	$P4_22_12$
Unit-cell parameters											
<i>a</i> (Å)	~52	54.97	51.55	51.25	54.46	78.27	79.13	N/D	75.22	N/D	75.37
<i>b</i> (Å)	~140	140.41	138.89	139.51	138.82	77.68	79.58	N/D	75.22	N/D	75.37
<i>c</i> (Å)	~179	180.23	179.10	179.54	179.13	81.00	80.58	N/D	99.77	N/D	100.83
β (°)	~95	90	90	90	90	89.57	90.04	N/D	90	N/D	90
Cell volume (Å ³)	1299100	1391100	1282300	1283700	1354200	485890	501230	N/D	564500	N/D	572780
Resolution (Å)	N/D	50–2.90 (2.95–2.90)	50–2.70 (2.75–2.70)	50–3.20 (3.26–3.20)	50–2.70 (2.75–2.70)	50–1.90 (1.93–1.90)	50–2.59 (2.64–2.59)	N/D	50–1.45 (1.48–1.45)	N/D	50–2.10 (2.14–2.10)
Unique reflections	N/D	29092	38424	20750	38466	76138	32300	N/D	96669	N/D	32650
Redundancy	N/D	6.0 (5.1)	6.4 (5.2)	8.0 (7.8)	6.3 (3.9)	3.4 (3.5)	3.7 (3.7)	N/D	7.2 (6.7)	N/D	12.3 (8.3)
Completeness (%)	N/D	92.4 (71.8)	98.1 (84.5)	99.7 (99.4)	98.2 (85.2)	99.6 (99.0)	99.9 (99.9)	N/D	99.9 (100.0)	N/D	100.0 (99.3)
<i>R</i> _{sym} (%)	N/D	12.1 (68.0)	10.1 (42.5)	16.6 (48.8)	13.1 (92.3)	18.2 (86.2)	15.6 (76.0)	N/D	6.8 (91.7)	N/D	13.7 (61.0)
<i>R</i> _{meas} (%)	N/D	18.5	13.1	21.5	16.5	17.1	16.0	N/D	9.4	N/D	15.5
<i>R</i> _{pim} (%)	N/D	11.4	7.0	11.3	7.0	9.3	5.8	N/D	3.5	N/D	4.3
$\langle I/\sigma(I) \rangle$	N/D	10.2 (1.0)	15.6 (2.8)	16.3 (4.1)	14.5 (1.0)	11.6 (1.1)	12.9 (1.3)	N/D	28.8 (2.3)	N/D	21.2 (3.2)
Mosaicity (°)	N/D	1.45	0.51	1.39	0.63	1.17	0.51	N/D	0.23	N/D	0.45

† Twinned. ‡ For one crystal out of many examined. Others were not single.

crystal, three or six crystals were picked up to be high-pressure cryocooled at each pressure.

The oil-coated crystals were high-pressure cryocooled as described by Kim *et al.* (2005), except using a high-pressure gas pump (Hydro-Pac) capable of reaching 400 MPa. The remaining high-pressure plumbing and components were commercially available and rated to at least 400 MPa. Three crystals could be pressurized simultaneously. Briefly, crystals were loaded into a high-pressure cryocooling apparatus consisting of commercial high-pressure plumbing and pressure transducers. The apparatus containing the crystals was then pressurized with dry helium gas to 200, 250, 300, 350 or 400 MPa, in less than one minute, and the crystal samples were equilibrated under pressure for 30 min. The magnetic constraints holding the mounted crystals were then released, allowing them to fall down a length of high-pressure tubing into a cold zone kept at liquid nitrogen temperature (77 K). After equilibration at liquid nitrogen temperature for 3 min, the helium pressure was released and the crystals were transferred to regular CrystalCaps (Hampton Research), under liquid nitrogen, for data collection. After the procedure, the high-pressure-cryocooled crystals can be handled at ambient pressure in the same manner as normal flash-cryocooled crystals for cryocrystallographic data collection.

2.3. Crystallographic data collection and processing

X-ray diffraction data were collected at the Cornell High Energy Synchrotron Source (CHESS) on beamlines F1 (ADSC Quantum-270 CCD detector) and A1 (ADSC Q-210 or PILATUS 6M detector). In all cases the detector face was perpendicular to the incident beam. The X-ray exposure time was 1–10 s with an oscillation angle of 1°. All data were collected at 100 K. The diffraction data were processed using the *HKL* package (Otwinowski & Minor, 1997); statistics of

data collection and processing from selected crystals are summarized in Table 1.

3. Results

3.1. Glutaminase C

The cuboid crystals of human GAC appeared to be single under an optical microscope, without concave or 'reentrant' crystal faces. However, the diffraction patterns of GAC crystals cryocooled at ambient pressure exhibited multiple lattices (Fig. 2*a*). Test data taken at room temperature were similar, showing that the disorder was intrinsic to the crystals, rather than resulting from the cryocooling process. Annealing and partial dehydration of crystals had no effect on the disorder. Images such as Fig. 2(*a*) indicate nonmerohedral twinning of a monoclinic unit cell, dimensions $52 \times 140 \times 179$ Å, $\beta = 95^\circ$ (Fig. 1*b*). Most diffraction data sets collected from crystals cryocooled at ambient pressure could not be indexed and integrated. Of more than 30 diffraction data sets, collected from more than 30 different crystals from different batches, only one instance was found of a processable set, which exhibited an orthorhombic symmetry (Table 1). To investigate the effect of high pressure on this disorder, GAC crystals were pressurized at 200, 250, 300, 350 or 400 MPa for 30 min each and then cooled to liquid nitrogen temperature. Three or six crystals were processed at each pressure, and X-ray diffraction data were collected. As shown in Figs. 2(*b*) and 2(*c*), the diffraction patterns of all crystals pressurized at 200 and 250 MPa still contained multiple lattices, suggesting they were still nonmerohedrally twinned crystals. None of the diffraction data sets collected on these crystals could be indexed. The diffraction patterns of the crystals pressurized at 300, 350 and 400 MPa each contained only one lattice (Figs. 2*d–f*), indicating that the crystals were single. The patterns were very

Figure 2

Diffraction patterns from native GAC. (a) The diffraction pattern from a crystal cryocooled at ambient pressure contains multiple lattices; the pattern suggests nonmerohedral twinning. Diffraction patterns from crystals high-pressure cryocooled at 200 MPa (b) and 250 MPa (c) are similar, still showing the twinning. Diffraction patterns of crystals high-pressure cryocooled at 300 MPa (d), 350 MPa (e) and 400 MPa (f) contain only one lattice, indicating that they are single crystals. Some of the split reflections in the diffraction patterns of nonmerohedrally twinned crystals are marked with red arrows.

Figure 3

Diffraction patterns from a GAC-inhibitor (#18) complex. (a) The diffraction pattern of a crystal cryocooled at ambient pressure contains multiple lattices; crystal appears to be twinned. Some of the split reflections are marked with red arrows. (b) The diffraction pattern of a crystal high-pressure cryocooled at 350 MPa contains only one lattice; the crystal appears single.

clean, without split spots or smearing. All of the diffraction data sets collected on these crystals were easily processed, with orthorhombic symmetry and low mosaicity, using standard *HKL-2000* (Otwinowski & Minor, 1997) (Table 1).

More than 60 inhibitors of GAC have been synthesized (unpublished data). Fifteen of them were selected to be studied crystallographically. Crystals of GAC in complex with these inhibitors were obtained using the co-crystallization method. As with the native GAC crystals, almost all crystals of these GAC-inhibitor complexes were nonmerohedrally twinned as shown by their diffraction patterns (Fig. 3*a*). The diffraction data sets collected from ambient cryocooled crystals could not be processed using standard *HKL-2000*. To overcome the twinning problem, three crystals of each GAC-inhibitor complex were pressurized at 350 MPa for 30 min and then cooled to liquid nitrogen temperature. Diffraction patterns showed that all these high-pressure cryocooled crystals were single (Fig. 3*b*); the data were easily processed using standard *HKL-2000*, with low mosaicity values (Table 1).

3.2. YjbM

These rod-shaped crystals, like those of GAC, appeared single under an optical microscope. However, the diffraction patterns of crystals cryocooled at ambient pressure contain more than one lattice, suggesting probable twinning (Fig. 4*a*). As with GAC, data collection at room temperature, or use of annealing or partial dehydration, did not reduce the disorder. In order to collect a data set that could be interpreted, more than 15 crystals from different batches were screened and five data sets were collected. However, only one out of these five data sets could be processed, and the mosaicity was high (Table 1). Nine YjbM crystals from different batches were high-pressure cryocooled at 200 MPa for 30 min. Diffraction patterns showed that these crystals were still twinned (Fig. 4*b*). Another nine crystals from different batches were high-pressure cryocooled at 350 MPa for 30 min. Diffraction patterns showed that these crystals were single (Fig. 4*c*); the data were easily processed using standard *HKL-2000* and showed low mosaicity (Table 1).

3.3. Lpg1496

These large thin plate-shaped crystals also appeared single under an optical microscope. However, diffraction patterns of crystals cryocooled at ambient pressure had the 'too-many-spots' appearance characteristic of very high mosaicity; the presence of split and smeared spots indicated some possible twinning as well (Fig. 5*a*). Over 20 diffraction data sets were collected on different crystals from different batches, and none of them could be indexed. Use of room temperature, annealing or partial dehydration did not help. Six lpg1496 crystals from different batches were high-pressure cryocooled at 200 MPa for 30 min. Diffraction patterns showed that these crystals were still disordered (Fig. 5*b*). Another six crystals from the same batches were high-pressure cryocooled at 350 MPa for 30 min and gave diffraction patterns indicating that they were well ordered (Fig. 5*c*). The data were easily

processed using standard *HKL-2000* and gave low mosaicity values (Table 1).

Crystals of Se-labeled lpg1496 were obtained under conditions similar to those of the native lpg1496. Like the native lpg1496 crystals, the Se-labeled lpg1496 crystals were also disordered. Nine Se-labeled lpg1496 crystals were high-pressure cryocooled at 350 MPa for 30 min and produced clean diffraction patterns indicative of well ordered single crystals (Fig. 5*d*). Single-wavelength anomalous diffraction data were collected and processed easily (Table 1).

4. Discussion

Previously reported benefits of high-pressure cryocooling include reduction of damage due to cryocooling (Kim *et al.*, 2005), ability to cryocool samples other than crystals mounted

Figure 4
Diffraction patterns from YjbM. (a), (b) Diffraction patterns of crystals cryocooled at ambient pressure (a) and 200 MPa (b) contain multiple lattices, suggestive of twinned crystals. Some of the split reflections in the diffraction patterns are marked with red arrows. (c) The diffraction pattern of a crystal high-pressure cryocooled at 350 MPa contains only one lattice, suggesting a single crystal.

Figure 5

Diffraction patterns from lpg1496. (a), (b) Diffraction patterns of crystals cryocooled at ambient pressure (a) and 200 MPa (b) clearly show disorder: too many spots for a single lattice as well as split and smeared spots (some labeled with red and blue arrows, respectively). (c), (d) Diffraction patterns of native (c) and Se-labeled (d) crystals high-pressure cryocooled at 350 MPa contain only one lattice, suggesting that these crystals are single.

in loops (Kim *et al.*, 2007; Lima *et al.*, 2014), stabilization of ligands (Albright *et al.*, 2006), trapping of gas molecules [noble gases for phasing (Kim *et al.*, 2006) as well as reactants in active sites (Domsic *et al.*, 2008)] and observation of pressure effects on molecular structure (Barstow *et al.*, 2009). We now add the use of HPC to reduce crystal disorder. When we used HPC with GAC crystals, we found that high pressure, 300–400 MPa, applied for 10–30 min, could transform nonmerohedrally twinned crystals to single crystals. Pressure below 250 MPa could not trigger this transformation, even if the pressurization time was prolonged to one hour. 300 MPa appears to be the critical point for the transformation: pressure higher than 300 MPa can remove the twinning disorder, while pressure lower than 300 MPa cannot. HPC at 200 MPa has been observed to induce a transformation of space group *P*23 (with poor resolution and high mosaicity) to space group *I*23 (with much improved resolution and mosaicity) in CpMV crystals at around 240 MPa. The requirement for at least 300 MPa in the GAC case could be due to the need for larger molecular shifts to transform the twinned monoclinic packing to untwinned orthorhombic. The transformation from twinned crystal to single crystal under high pressure completes rapidly; at 350 MPa, 10 min are enough for GAC crystals to transform from twinned crystals to single crystals. After being pressurized at 300–400 MPa for 10–30 min, GAC crystals were cooled to liquid nitrogen temperature in less than one second

(Kim *et al.*, 2013) and then the high pressure was released. At this temperature, the high-pressure conformation is locked in, and the GAC crystals could retain their single status for at least one month.

Is GAC a unique case which is particularly susceptible to pressure-induced ordering? To answer this question, we investigated two additional proteins, YjbM and lpg1496, which were the subjects of collaborative studies and presented difficulties in data collection. As observed for GAC, crystals of these two proteins are almost always disordered at ambient pressure. YjbM has a monoclinic cell with *a*, *b* and *c* all very similar, and a β angle very close to 90° (Table 1), with many possibilities for nearly pseudomerohedral twinning. Not surprisingly, diffraction patterns show multiple lattices and split spots (Fig. 4a). Diffraction patterns from lpg1496 clearly indicate very high mosaicity and possibly some form of twinning; the patterns are not indexable and the unit cell is unclear (Fig. 5a). After pressurization at 300–400 MPa for 10–30 min, both types of crystals were transformed to single untwinned forms (Figs. 4c, 5c and 5d). Pressure below 250 MPa did not trigger this transformation, even when the time at high pressure was increased to one hour. Therefore, GAC is not a unique case, and reduction in disorder by high pressure may be common in macromolecular crystals; further experiments are needed to verify this hypothesis. These two cases of YjbM and lpg1496 also support the hypothesis that a pressure of order 300 MPa is required to transform highly mosaic and/or nonmerohedrally twinned crystals to well ordered single crystals.

We do not claim to understand the detailed mechanism whereby high pressure induces better ordering in the crystals, but the overall principles are clear. Water exists in distinct pools within the sample, including within nanovoids in each protein, as crystallographically bound water to protein surfaces, as unbound water in water channels between proteins within each mosaic block, as unbound water between mosaic blocks and as bulk water surrounding the crystal. In equilibrium there is no net movement of water between the pools. However, the compressibilities differ for each of these water pools, as well as for the protein molecules themselves. Therefore, as pressure is applied water will re-equilibrate between the pools. If, for example, the net movement of water is from volumes between mosaic blocks, then there is a thermodynamic drive for the mosaic blocks to grow and/or more closely align.

Fig. 1 illustrates such a possibility as a driving mechanism for the ordering effect of pressure: reduction of the voids between mosaic blocks or twin domains decreases the volume of the crystal. Although the transformation in Fig. 1(b) appears dramatic, it can in fact be accomplished by a minor change in the packing of the macromolecules. Examples of the same protein with different crystal packings abound (Gallagher & Croker, 1994). Note that the ordering can occur without a reduction in the unit-cell volume (Table 1). The transformation also typically has little or no effect on the structure of the protein itself. Fig. 6 shows a superposition of the crystal structures of GAC cryocooled at 350 MPa and at ambient pressure (columns 3 and 2, respectively, in Table 1; the structure from column 3 has been deposited in the Protein

Data Bank with ID 5d3o). The two structures are nearly identical, with an RMSD between all non-hydrogen atoms of 0.39 Å, well within experimental error for coordinates at this resolution, and no changes in crystal packing are visible. At ambient pressure GAC has two flexible domains (N-terminal domain, residues 71–142; C-terminal domain, residues 536–598) that are not visible in the crystal structure; the same is true with HPC at 350 MPa.

Recently software has been developed to detwin merohedrally or pseudomerohedrally twinned macromolecular crystals, e.g. *DETWIN* in the *CCP4 Suite* (Winn *et al.*, 2011). However, for nonmerohedrally twinned macromolecular crystals, if an indexing routine cannot identify a major lattice, new crystallization conditions giving untwinned crystals must be sought. Similarly, if all crystals of a protein are highly mosaic and diffract only to low resolution, new crystallization conditions must be explored. This is frequently a time-consuming and uncertain process. Sometimes no crystallization conditions giving well ordered crystals for the given protein can be found, and it is necessary to resort to modification of the protein [truncation or point mutation(s)] by genetic engineering. Here we find that HPC using a pressure above 300 MPa can remove some disorder, including nonmerohedral twinning, making it possible to collect usable diffraction data from previously intractable crystals. In the case of GAC, tens of crystals were screened, and only one crystal was found to be 'less twinned' and produced usable diffraction data. Hence, in order to solve the crystal structures of GAC in complex with 15 different inhibitors without high-pressure cryocooling, hundreds of crystals might need to be screened, and it is difficult to grow tens of large crystals of each complex. Therefore, in this case, HPC is very useful; for each complex, we anticipate that only a few crystals will need to be pressurized in order to collect usable diffraction data.

Note that use of HPC is not the only means of obtaining structures of the three proteins tested. A structure of human GAC (DeLaBarre *et al.*, 2011; PDB entry 3uo9) was obtained using protein expressed in SF9 insect cells rather than *E. coli*; a monoclinic cell similar to that in column 1 of Table 1, but untwinned, was observed (the authors do not report whether the crystal used was typical or the best of a variable lot). Additionally, as reported in Table 1, we occasionally obtained a usable orthorhombic crystal using ambient-pressure cryocooling. For YjbM also, a few good crystals appeared among the many poor ones (Table 1 column 6, and Steinchen *et al.*, 2015; PDB entry 5dec). For lpg1496, a recent publication (Wong *et al.*, 2015) reported structures of a number of differently truncated versions, including one (PDB entry 5bu0) just five residues longer than the form we used (Table 1 column 9). Nevertheless, the use of HPC can clearly improve the success rate for obtaining useful data from 'difficult' crystals. So far, we have only worked with three proteins exhibiting the type of disorder discussed above, and we have obtained good results with all of them; we are currently seeking additional proteins which produce primarily, or solely, disordered crystals, to determine whether HPC for reducing disorder will be broadly applicable.

Figure 6
Superposition of the crystal structures of GAC at 350 MPa (red) and at ambient pressure (green). These two structures have no obvious difference, with an RMSD between atoms of 0.39 Å.

5. Conclusions

We have demonstrated the effectiveness of high-pressure cryocooling at a pressure of 300 MPa or above in improving crystal quality by reducing disorder. In multiple cases, the technique has enabled structure solution from crystals that would otherwise have been discarded. Further work is needed to determine how often this approach will succeed, but it is clear that HPC is a tool which should be considered when crystals are consistently nonmerohedrally twinned or of very high mosaicity. HPC, with pressures up to 400 MPa, is available as a service at CHESS (<http://www.macchess.cornell.edu>). HPC up to 200 MPa is available at several locations around the world, e.g. beamline ID-23 at ESRF (<http://www.esrf.eu/UsersAndScience/Experiments/MX>) and beamline 2D at the Pohang Light Source (<http://pal.postech.ac.kr/paleng/bl/2D>), and a commercial apparatus is sold by Advanced Design Consulting (<http://adc9001.com>); future upgrades to 400 MPa would be straightforward.

Acknowledgements

This work is based on research conducted at the Cornell High Energy Synchrotron Source (CHESS), which is supported by the National Science Foundation and the National Institutes of Health/National Institute of General Medical Sciences under NSF award DMR-1332208, using the Macromolecular Diffraction at CHESS (MacCHESS) facility, which is supported by award GM-103485 from the National Institute of General Medical Sciences, National Institutes of Health. We thank Jennifer Wierman and Mark Tate for assistance in high-pressure cryocooling experiments.

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