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Supercooled liquid-like solvent in trypsin crystals: implications for crystal annealing and temperature-controlled X-ray radiation damage studies

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The study of temperature-dependent physical changes in flash-cooled macromolecular crystals is pertinent to cryocrystallography and related issues such as crystal annealing, X-ray radiation damage and kinetic crystallography. In this context, the unit-cell volume of flash-cooled trigonal and orthorhombic trypsin crystals has been monitored upon warming from 100 to 200 K and subsequent re-cooling to 100 K. Crystals of both forms were obtained under the same crystallization conditions, yet they differ in solvent content and channel size. An abrupt non-reversible unit-cell volume decrease is observed at 185 K in orthorhombic and at 195 K in trigonal crystals as the temperature is increased; this result is consistent with ultra-viscous solvent leaving the crystals. Concomitant appearance of ice rings in the diffraction patterns suggests that the transported solvent forms crystalline ice. These results demonstrate that solvent in flash-cooled protein crystals is liquid-like near its crystallization temperature, as has been proposed, yet controversially discussed, for the case of pure water. The use of mineral oil prevents the unit-cell volume decrease in trigonal but not in orthorhombic crystals. The observation of liquid-like solvent has implications in the development of annealing protocols and points a way to the rational design of temperature-controlled crystallographic studies that aim either at studying specific radiation damage or at trapping enzymatic intermediate states.

Keywords: cryocrystallography; crystal annealing; ultra-viscous solvent; radiation damage; kinetic crystallography.

1. Introduction

Modern protein X-ray crystallography predominantly makes use of cryocooled loop-mounted crystals (Teng, 1990). The temperature at which X-ray data are collected is typically 100 K (Garman & Doublie, 2003). Owing to the limited radical diffusion at cryotemperatures, the lifetime of protein crystals in the X-ray beam is greatly enhanced, an important issue, especially when working with radiation from synchrotron sources (Hope, 1988; Garman, 2003). The physical aspects of flash-cooled protein crystals remain poorly understood. However, the success of crystal annealing protocols that can improve diffraction quality of cryocooled protein crystals (Harp *et al.*, 1998; Yeh & Hol, 1998), and the description of temperature-dependent radiation damage (Weik, Ravelli *et al.*, 2001; Ravelli *et al.*, 2002; Teng & Moffat, 2002), have triggered a renewed interest in studying the behavior of protein crystals at cryotemperatures (Weik, Kryger *et al.*, 2001; Juers & Matthews, 2001, 2004; Kriminski *et al.*, 2002; Parkin & Hope, 2003; Weik, 2003). Insight into the low-temperature behavior of flash-cooled protein crystals greatly relies on the extended body of work carried out over the past few decades in the field of pure water; this work will be briefly summarized.

Crystallization of water can be bypassed by rapid cooling from the liquid phase or by low-pressure vapor-deposition on a cold plate. Hyper-quenched glassy water (HQGW) is formed by the former, and amorphous solid water (ASW) by the latter method [for reviews see Mayer (1991) and Mishima & Stanley (1998)]. Alternatively, pressure amorphization at 77 K and 10 kbar of hexagonal ice leads to high-density amorphous ice (HDA), which transforms into low-density amorphous ice (LDA) at zero pressure and 117 K (Mishima *et al.*, 1984). The

transition from HDA (density of 1.17 g cm^{-3} at zero pressure) to LDA (0.94 g cm^{-3}) is accompanied by a volume increase of more than 20% (Mishima *et al.*, 1985). LDA, ASW and HQGW have similar structures and densities (Mishima & Stanley, 1998). It has been widely accepted that these three forms undergo a glass transition upon warming (at 136 K for ASW and HQGW, and at 129 K for LDA) (McMillan & Los, 1965; Johari *et al.*, 1987; Mayer, 1991), transforming into an ultra-viscous liquid (Mishima & Stanley, 1998) that crystallizes into cubic crystalline ice at around 150 K (Mayer, 1991). Cubic ice transforms into hexagonal ice upon further warming at 186 K (McMillan & Los, 1965). The observation of long-range translational diffusion of water molecules at 150 K provided evidence for the existence of ultra-viscous water (Smith & Kay, 1999), which has been shown to coexist with crystalline ice up to 210 K (Jenniskens *et al.*, 1997). Recently, however, the existence of ultra-viscous water has been questioned (Yue & Angell, 2004), and vitreous water has been proposed to remain in the glassy state until it crystallizes at 150–160 K (Velikov *et al.*, 2001).

The transfer of knowledge on the behavior of pure water to that of the solvent in low-temperature protein crystals is useful, although such an extrapolation is hampered by the controversies mentioned above. Furthermore, the solvent is confined within protein crystals and its composition differs from pure water. The confinement of water alters its low-temperature behavior compared with bulk water, mainly owing to the interaction of water molecules with the surface of the confining medium [for reviews see Teixeira *et al.* (1997), Dore (2000) and Bellissent-Funel (2001)]. As a result, the hydrogen bonding is enhanced, the nucleation temperature depressed and the viscosity increased. The addition of cryoprotectants further increases the viscosity of the crystal solvent. As a consequence, solvent in protein crystals can be successfully vitrified at cooling rates obtained with cold-nitrogen gas streams (50–500 K s^{-1}; Teng & Moffat, 1998; Walker *et al.*, 1998; Snell *et al.*, 2002), whereas cooling rates of more than 10^5 K s^{-1} are required to vitrify micrometer-sized droplets of pure water (Johari *et al.*, 1987). Upon flash-cooling, the unit-cell volume of a protein crystal contracts by 2–7% (Juers & Matthews, 2001, and references therein). The protein molecules themselves contract by only 1–3%, and hence most of the unit-cell contraction is due to contraction of the solvent part of the crystal, suggesting that lattice repacking occurs upon flash-cooling (Juers & Matthews, 2001). Additionally, it has been suggested that solvent transport out of the crystal, or into macroscopic pockets within the crystal, occurs during flash-cooling, thus partially accounting for the observed unit-cell contraction (Juers & Matthews, 2001, 2004). The unit-cell behavior upon heating after flash-cooling has been studied in only a very limited number of cases (Weik, Kryger *et al.*, 2001; Parkin & Hope, 2003). If the solvent is confined within small channels (*e.g.* 10 Å in diameter), the unit-cell volume increases linearly in the studied temperature range between 100 and 180 K. For trigonal acetylcholinesterase and β2gpI crystals with large channels (> 60 Å in diameter), an abrupt increase in unit-cell volume has been observed at 155 K. This has been attributed to the water fraction of the solvent crystallizing within the channels, providing circumstantial evidence that a glass transition must have occurred at or below 155 K (Weik, Kryger *et al.*, 2001). A small increase in unit-cell volume at 160–165 K has been observed for concanavalin A crystals with 30 Å channels, consistent with the solvent either transforming from one amorphous form to another or forming crystalline ice within the channels (Parkin & Hope, 2003). However, no evidence has been presented yet that solvent within flash-cooled protein crystals transforms into an ultra-viscous liquid at temperatures around its crystallization temperature, as has been suggested for pure water (see above).

We present here data on the unit-cell behavior of flash-cooled orthorhombic and trigonal trypsin crystals upon warming to 200 K and subsequent re-cooling to 100 K. The two forms have identical solvent compositions, yet the sizes of the channels that run straight through the crystals are slightly different (cross sections of 16 Å × 21 Å in the orthorhombic and 12 Å × 16 Å in the trigonal crystals). An abrupt unit-cell volume decrease sets in during heating at 185 and 195 K for the crystals with the larger (orthorhombic) and the smaller (trigonal) channels, respectively, and a concomitant appearance of ice rings is observed in the diffraction patterns. Our data are consistent with solvent leaving the crystal at the water crystallization temperature, hence providing evidence for a transformation into an ultra-viscous liquid. Employing mineral oil, the cell volume decrease can be prevented for crystals with the smaller solvent channels but not for those with the larger channels. The results suggest an extension of already existing crystal annealing protocols (Harp *et al.*, 1998, 1999; Yeh & Hol, 1998; Kriminski *et al.*, 2002) and this is detailed in the discussion. Furthermore, the sudden drop in solvent viscosity is pertinent to temperature-dependent radiation damage studies and kinetic protein crystallography.

2. Materials and methods

2.1. Crystallization and flash-cooling

Bos taurus trypsin (23 kDa) was purchased from Sigma and used without further purification. Trigonal ($P3_121$) and orthorhombic ($P2_12_12_1$) crystals were grown at room temperature in 23–25% (*w*/*v*) polyethylene glycol (PEG) 8000, 0.2 *M* ammonium sulfate, 0.1 *M* Tris buffer pH 8 and 100 m*M* benzamidine (inhibitor). An initial protein concentration of 15 mg ml^{-1} was employed (Kurinov & Harrison, 1994). Trigonal and orthorhombic crystals contain 18 and 42% solvent, respectively. Crystals of the two forms were about 300 µm × 50 µm × 50 µm in dimension, grew in the same drops and could not clearly be distinguished by visual inspection. Crystals were transferred for ~20 s in 24% (*w*/*v*) PEG 8000, 0.2 *M* ammonium sulfate, 0.1 *M* Tris buffer pH 8 and 15% (*v*/*v*) glycerol and mounted directly in a cryoloop or transferred into a drop of mineral oil, in order to remove excess solvent, before being loop-mounted. For flash-cooling, the N_2 gas stream of a cooling device operating at 100 K (600

series, Oxford Cryosystems, Oxford, UK) was blocked swiftly with a piece of card; the crystal was placed onto the goniometer head and the card was then removed.

2.2. Data collection and processing

A Nonius KappaCCD diffractometer, mounted on a sealed-tube source producing Cu $K\alpha$ radiation (λ = 1.54 Å), was employed for the X-ray diffraction experiments. Prior to initiation of a warming–cooling procedure, accurate unit-cell parameters were determined for each crystal at 100 K. To this end, the φ–χ method was applied ($\Delta\varphi$ = 2°, $\Delta\chi$ = 10°; Duisenberg *et al.*, 2000) by collecting images at four different φ positions, 90° apart, so as to provide input data for the auto-indexing program *DirAx* (Duisenberg, 1992). The resulting orientation matrix was then continuously refined, using *DENZO* (Otwinowski & Minor, 1997), against the data collected at different temperatures during the warming–cooling procedure described below. Unit-cell dimensions at 100 K were typically a = 54 Å, b = 58 Å and c = 67 Å for orthorhomic, and $a = b$ = 55 Å and c = 108 Å for trigonal crystals. The temperature of the cooling device was changed from 100 to 200 K and back to 100 K in 5 K increments at a rate of 360 K h^{-1}. The temperatures reported in this manuscript are those displayed by the cooling device controller, which are in general about 2 K lower than the actual temperature at the crystal position (Weik, Ravelli *et al.*, 2001). At each temperature, a data set was collected consisting of ten images with an oscillation angle $\Delta\varphi$ of 0.2° and an exposure time of 90 s per image. Each data set took 19 min to record. In order to monitor crystalline ice formation, intensities in the diffraction pattern were radially integrated and were summed in the 2.30–2.19 Å and the 3.79–3.99 Å resolution shells. The former includes the superimposed rings of the (110) reflection (2.25 Å) of hexagonal ice and the (220) reflection (2.25 Å) of cubic ice, and the latter includes a powder ring originating from the (100) reflection (3.91 Å) of hexagonal ice.

3. Results

Fig. 1 shows the unit-cell volume relative to that at 100 K of flash-cooled (cryoprotected but not transferred to oil) orthorhombic (Fig. 1*a*) and trigonal (Fig. 1*b*) trypsin crystals upon warming from 100 to 200 K and subsequent cooling back to 100 K. Upon warming, the unit-cell volume of orthorhombic crystals increases by 1% in the temperature range 100–170 K, displays a slight decrease between 170 and 185 K, and drastically decreases between 185 and 200 K. The rate of decrease remains high during recooling from 200 to 185 K and reduces in the 185–100 K temperature window. Powder diffraction rings corresponding to d-spacings of 2.25 and 3.91 Å begin to form at 170−185 K upon warming and continue to increase at temperatures above 185 K, both upon warming to 200 K and subsequent re-cooling. The 2.25 Å ring originates from cubic or hexagonal ice, and the 3.91 Å ring from hexagonal ice only. The unit-cell volume decreases by 9% between 170 K (during heating) and 100 K (after re-cooling). Employing the same experimental protocol, the behavior of the unit-cell volume of trigonal crystals is qualitatively similar yet quantitatively different from that of orthorhombic crystals. The volume increases upon warming in the temperature range 100–195 K and decreases above 195 K and upon cooling back to 100 K. The unit-cell volume decreases by 2% between 195 K upon warming and subsequent re-cooling to 100 K. Ice rings at 2.25 and 3.91 Å appear at about 185 K upon warming and increase in intensity until the maximum temperature of 200 K is reached. The results of the experiment presented in Fig. 1(*a*) have been reproduced with three different crystals. That in Fig. 1(*b*) has been carried out only once, owing to the many trials necessary to identify crystals of the rare trigonal form.

The results in Fig. 2 were obtained with crystals that were transferred to mineral oil after cryoprotection. The unit-cell behavior of orthorhombic crystals during the warming–

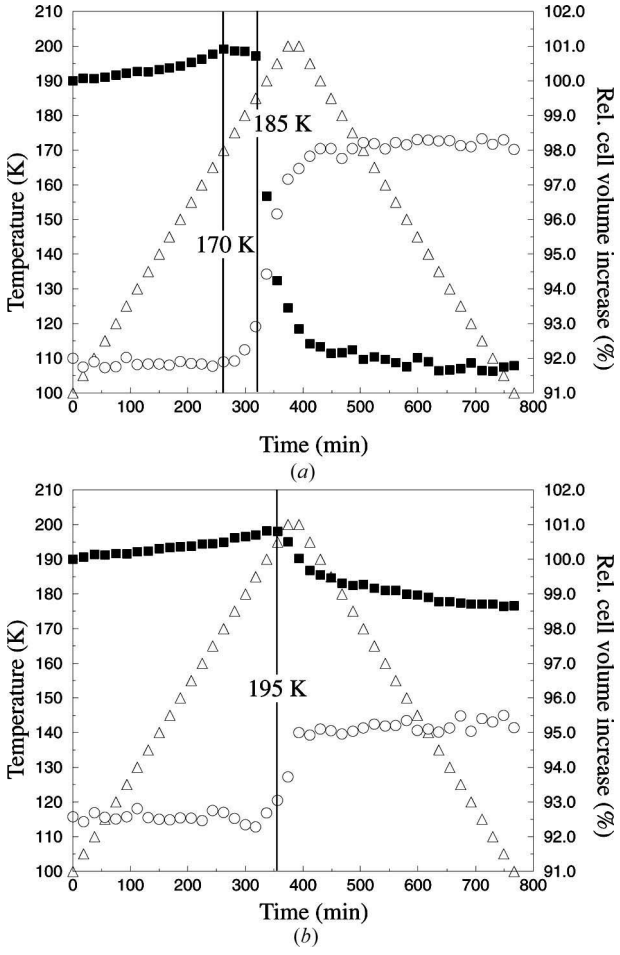

Figure 1
Relative unit-cell volume (closed squares), temperature (open triangles) and integrated ice-ring intensities (open circles) as a function of experimental time for (*a*) an orthorhombic and (*b*) a trigonal trypsin crystal that were cryoprotected without subsequent transfer to mineral oil. The ice intensities were summed up in the 2.3–2.19 Å resolution range and include the (110) reflection of hexagonal and the (220) reflection of cubic ice. Ice intensities do not refer to either of the y axes, are on an arbitrary scale and were zero at 100 K prior to initiation of the warming–cooling procedure.

cooling procedure (Fig. 2*a*) is similar to that presented in Fig. 1(*a*) of cryoprotected crystals without oil. At 185 K upon heating, the unit-cell volume starts to decrease drastically to reach a 9% lower value at 100 K after re-cooling. Only the 2.25 Å ice ring forms at 185 K, whereas the 3.91 Å ring remains absent, indicating that only cubic ice is formed. The unit-cell volume of the trigonal form, however, changes reversibly if cryoprotected crystals are additionally transferred to mineral oil (Fig. 2*b*). The unit-cell volume increases almost linearly by about 1% upon heating from 100 to 200 K and contracts by 1% during the subsequent cooling to 100 K, without any apparent drastic decrease at 195 K. No ice rings are visible in the diffraction pattern during the entire heating–cooling procedure. The experiments presented in Fig. 2 have been carried out only once.

The diffraction quality as a function of temperature was qualitatively assessed by monitoring the mosaicity and the number of partially recorded reflections with $I/\sigma(I) > 10$ (data not shown). For orthorhombic crystals with and without oil, the diffraction quality remained constant up to 185 K upon heating and then abruptly deteriorated. In contrast, trigonal crystals with and without oil preserved their diffraction quality throughout the entire temperature range studied. If the diffraction quality were to be examined more extensively in future experiments, more than a total oscillation range of 2° and several wedges 90° apart should be recorded.

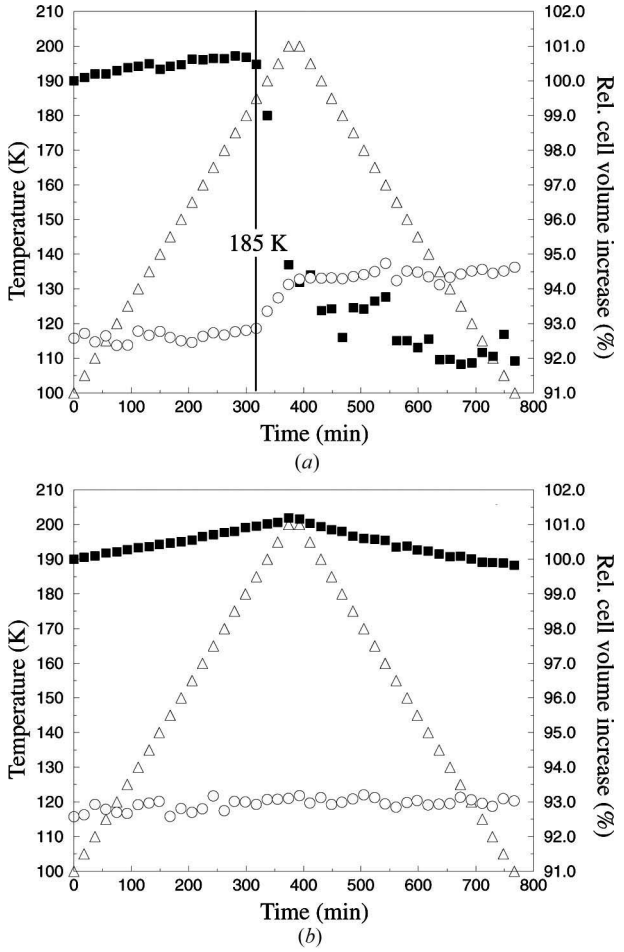

Figure 2
Relative unit-cell volume (closed squares), temperature (open triangles) and integrated ice-ring intensities (open circles) as a function of experimental time for (*a*) an orthorhombic and (*b*) a trigonal trypsin crystal that were cryoprotected and transferred to mineral oil. The ice-ring intensities (2.3–2.19 Å) do not refer to either of the *y* axes, are on an arbitrary scale and were zero at 100 K prior to initiation of the warming–cooling procedure.

4. Discussion

4.1. Super-cooled liquid-like solvent in protein crystals

We monitored unit-cell volume changes of flash-cooled orthorhombic and trigonal trypsin crystals upon warming from 100 to 200 K and subsequent re-cooling to 100 K, with the aim of extending the number of different protein crystals studied previously (Weik, Kryger *et al.*, 2001; Parkin & Hope, 2003). The solvent in both crystal forms is of identical composition, yet the sizes of continuous channels (Fig. 3) and the solvent contents are different. If the crystals are not transferred to mineral oil, the unit-cell volume abruptly decreases during warming at 185 K for the orthorhombic and at 195 K for the trigonal crystals (Fig. 1). Transfer to mineral oil prevents the abrupt unit-cell volume decrease for trigonal but not for orthorhombic crystals (Fig. 2). Solvent transport out of the unit cells or an increase in solvent density within the unit cells could explain the observed decrease. However, an increase in solvent density, being caused by, for example, a transition of the water fraction from LDA (0.94 g cm^{-3}) to HDA (1.17 g cm^{-3}), seems highly unlikely since HDA is formed under pressure and transforms to LDA upon heating (Mishima *et al.*, 1984). Even a transformation of LDA to an amorphous form with only a density close to that of liquid water (1 g cm^{-3}) would not account for the observed cell volume decrease of 9% in the case of orthorhombic crystals. Furthermore, if changes in solvent density were at the origin of the observed volume decrease, non-penetrating mineral oil would not be able to prevent it in the case of trigonal crystals. Therefore, we attribute the abrupt cell volume decrease to solvent leaving the unit cells. Where does the solvent go? It might either accumulate in grain boundaries within the crystal (Kriminski *et al.*, 2002) or leave the crystal to accumulate at the surface (Juers & Matthews, 2004). Since the cell volume decrease can be suppressed by mineral oil in trigonal crystals ('sealing' of the small channels), it is most likely that the solvent is transported out of the crystal when the cell volume decreases. The simultaneous appearance of ice rings and cell volume decrease is strongly suggestive of solvent forming crystalline ice at the crystal surface. On the basis of the arguments put forward above, we conclude that solvent in flash-cooled trypsin crystals has transformed into an ultra-viscous liquid at its crystallization temperature (185 K in orthorhombic and 195 K in trigonal trypsin crystals), since the solvent exhibits long-range translational diffusion. This corroborates the suggestion of Mishima & Stanley (1998) that amorphous bulk water exhibits liquid-like properties when it crystallizes. The question as to whether the solvent glass

transition in trypsin crystals occurs at or below the crystallization temperature remains open and might be answered by temperature-derivative fluorescence microspectrophotometry (Weik *et al.*, 2004). A similar observation of liquid-like water being transported out of a confined space at 200 K has been made with flash-cooled stacks of purple membranes (Weik *et al.*, 2005).

The presence of non-penetrating oil reduces unit-cell volume changes of protein crystals during cyclic thawing and freezing between 100 K and room temperature (Juers & Matthews, 2004). It has been concluded that the oil forms a kinetic barrier to water transport during the melting phase. Our observation of abrupt cell volume changes being suppressed in trigonal crystals with oil reinforces this reasoning. We point out that, even in the presence of oil, solvent in trigonal-crystal channels (12 Å × 16 Å) is most likely ultra-viscous above 195 K, even though solvent transport and crystallization are suppressed. However, oil is apparently not capable of preventing solvent draining in the orthorhombic crystal form with somewhat larger channels (16 Å × 21 Å). It is interesting to note that cubic ice forms in the case of orthorhombic crystals with oil, whereas hexagonal and possibly cubic ice forms in the cases of orthorhombic and trigonal crystals without oil. Water confined in small pores has been generally accepted to form only cubic ice (Dore, 2000), suggesting that the solvent creates such pores in the oil layer when it is transported to the crystal surface.

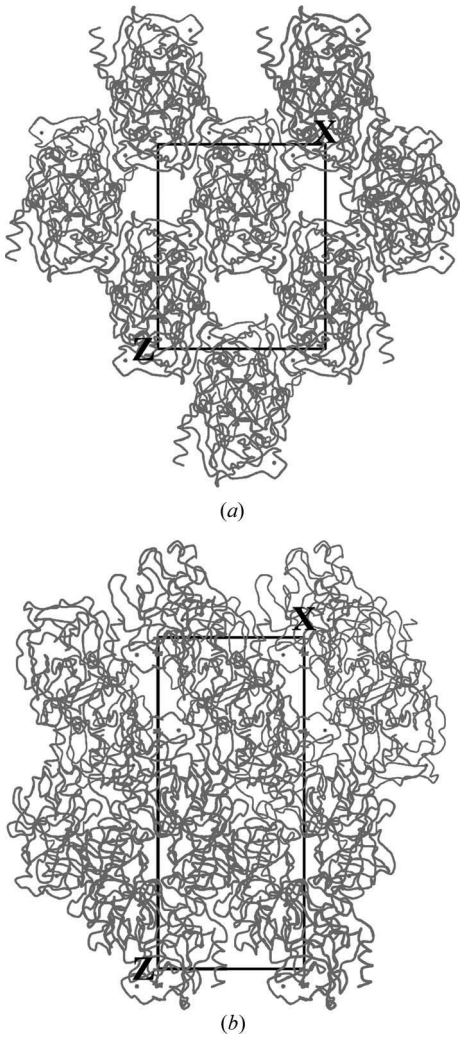

Figure 3
Packing diagram viewed down the *y* axis for (*a*) orthorhombic and (*b*) trigonal trypsin crystals. Structural data of the orthorhombic and the trigonal crystal forms are from PDB entries 1utn and 1uto, respectively (Leiros *et al.*, 2004). The unit-cell dimensions at 100 K are about $a = 54$ Å, $b = 57$ Å and $c = 66$ Å for the orthorhombic crystal form and $a = b = 55$ Å and $c = 107$ Å for the trigonal crystal form. Solvent channels along the *y* axis in the orthorhombic crystals are about 16 Å × 21 Å in size. The largest continuous solvent channels along the *y* axis in the trigonal crystals have dimensions of about 12 Å × 16 Å. The figure was created with *MOLSCRIPT* (Kraulis, 1991).

What drives the solvent out of the crystal? Confinement of solvent within 20 Å channels suppresses ice nucleation. We speculate that crystallization at the surface decreases the concentration of ultra-viscous water at the crystal surface, hence increasing the concentration of solutes and consequently sucking water out of the channels, in a process comparable to freeze-induced dehydration of biological cells at sub-zero temperatures (Gleeson *et al.*, 1994). Similar observations have been made for inter-membrane water in stacks of planar lipid membranes (Gleeson *et al.*, 1994) and purple membranes (Lechner *et al.*, 1998). Upon slow-cooling to below 273 K, the lamellar spacing (the repeat distance comprising the membrane and water-layer thickness) decreased abruptly, concomitant with the appearance of crystalline ice diffraction peaks. It has been concluded that water leaves the inter-membrane space to form crystalline ice outside the stacks. It is known that the chemical potential of inter-membrane water depends on the lamellar spacing. Furthermore, the temperature dependence of this chemical potential is different from that of crystalline ice. Upon changing the temperature, therefore, the lamellar spacing adjusts until the chemical potential values of inter-membrane water and crystalline ice are equalized (Gleeson *et al.*, 1994). A similar reasoning might explain why the solvent drains from the two-dimensional confinement in channels of orthorhombic and trigonal trypsin crystals.

From the present work and recent papers (Weik, Kryger *et al.*, 2001; Juers & Matthews, 2001, 2004; Kriminski *et al.*, 2002; Parkin & Hope, 2003) a possible view emerges concerning the dependence on channel sizes of solvent behavior in flash-cooled protein crystals upon warming to temperatures above 100 K. Solvent in small channels (∼10 Å; Weik, Kryger *et al.*, 2001) is mostly associated with the protein surface, is not transported during melting and does not crystallize. Larger channels (∼20 Å; this study) may force a fraction of the solvent to leave the crystal in order to form crystalline ice at the surface, which results in a decrease in unit-cell volume. For channel sizes of ∼30 Å (Parkin & Hope, 2003), the solvent either transforms into another amorphous solid or forms crystalline ice that does not give rise to sharp detectable ice rings in the diffraction pattern. For channels of 60 Å and larger, the solvent has enough space to crystallize within the protein crystal, resulting in a unit-cell volume increase (Weik, Kryger *et al.*, 2001). We suggest, but cannot prove, that solvent

in all flash-cooled protein crystals with channels larger than 20 Å transforms into an ultra-viscous liquid upon heating at or just below its crystallization temperature. The latter depends on the channel size and on solvent composition and has been reported to take place at temperatures between 155 and 195 K for the few cases studied. For a given solvent composition, a reduction in channel size increases the crystallization temperature (and therefore the glass transition temperature) as illustrated by, for example, the trigonal and orthorhombic trypsin crystals employed in this study. More cases need to be investigated before the presented view can be accepted for protein crystals in general. Furthermore, crystals that present only solvent cavities and lack any continuous channels should be studied.

4.2. Implications for crystal annealing

Several crystal annealing protocols have been developed over recent years that can improve diffraction from flash-cooled protein crystals. The most widely used are macromolecular crystal annealing (MCA; Harp *et al.*, 1998, 1999) and flash-annealing (Yeh & Hol, 1998) techniques. In the former, the flash-cooled protein crystal is warmed to room temperature and placed for 3 min in a drop of cryoprotectant before being loop-mounted and flash-cooled again. In the latter, the crystal remains in the loop and the cold stream is blocked for a few seconds; the crystal is then flash-cooling again. Both techniques have been successfully applied in several cases (reviewed by Hanson *et al.*, 2003) and can reduce mosaicity, improve the resolution limit and eliminate crystalline ice formed during non-ideal crystal-handling. Recent work has started to shed light on the physical processes involved in crystal annealing (Kriminski *et al.*, 2002; Juers & Matthews, 2001, 2004). Mismatch between the thermal contraction of the protein lattice and the cryoprotectant has been proposed to play a major role in diffraction degradation that often accompanies flash-cooling. The resulting lattice stress is reduced upon annealing, leading to larger better ordered domains and, hence, improved diffraction quality (Kriminski *et al.*, 2002). Furthermore, solvent transport into or out of the crystal has been shown to occur during the thawing phase, which modifies the cryoprotectant concentration (Juers & Matthews, 2004). Consequently, the success of crystal annealing has been ascribed to an improved match of lattice and solvent contraction, since the latter depends on the solvent concentration (Kriminski *et al.*, 2002; Juers & Matthews, 2004). It should be noted, however, that annealing is likely to be useful only in cases where cryoconditions have not been optimized (Mitchell & Garman, 1994).

A third annealing protocol is termed constant temperature annealing (CTA), in which the crystal is warmed to below room temperature (Weik, Kryger *et al.*, 2001; Kriminski *et al.*, 2002; Parkin & Hope, 2003). Weik, Kryger *et al.* (2001) suggested that annealing slightly below the solvent crystallization temperature might lead to release of lattice stress. Indeed, the highest-resolution data set of *Torpedo californica* acetylcholinesterase (*Tc*AChE, PDB code 1ea5) has been collected at 155 K, the temperature at which the solvent starts to form crystalline ice in these crystals. Kriminski *et al.* (2002) carried out a careful study in which they examined the effect of CTA at 233 and 253 K on the diffraction quality of tetragonal hen egg-white lysozyme (HEWL) crystals. The diffraction quality improved upon annealing at both temperatures. It is interesting to note that a fraction of the water in tetragonal HEWL crystals has been reported to become mobile at 240–250 K (Morozov & Gevorkian, 1985), suggesting that successful annealing at 253 K might involve solvent transport as well. CTA of concanavalin A crystals at 170 K, *i.e.* above the solvent transition detected at 160–165 K, led to a small improvement in diffraction quality as assessed by comparison of two data sets collected at 130 K before and after annealing (Parkin & Hope, 2003). Since solvent transport is involved in successful crystal annealing, and since we demonstrated liquid-like properties of solvent in trypsin crystals, we renew the suggestion by Weik, Kryger *et al.* (2001) that annealing at or just below the crystallization temperature may lead to improved diffraction quality. In the particular case of trypsin crystals, we anticipate annealing to be beneficial for orthorhombic crystals at temperatures between 170 and 185 K (where ice formation is still limited, yet the cell volume has already started to decrease) and for trigonal crystals in oil at or slightly above 195 K. In the latter case, oil prevents solvent transport and crystalline ice formation at the crystal surface, but the solvent most probably still exhibits liquid-like properties within the channels. In many cases, a complete data set can be collected at the solvent crystallization temperature without the appearance of ice rings when ice formation is slow on the time scale of data collection. However, if synchrotron sources are employed, the risk of increased radiation damage due to increased mobility of radicals has to be considered as well. This case will be described in the next section.

4.3. Implications for temperature-dependent radiation damage studies and kinetic protein crystallography

Synchrotron radiation can produce highly specific chemical and structural damage to protein structures (Burmeister, 2000; Ravelli & McSweeney, 2000; Weik *et al.*, 2000; Schrøeder Leiros *et al.*, 2001). The most prominent effects are breakage of disulfide bonds and decarboxylation of acidic residues. Specific damage is temperature dependent, as has been shown by a series of data collections at 100 and 155 K in trigonal *Tc*AChE crystals (Weik, Ravelli *et al.*, 2001). Since 155 K is the temperature at which the solvent in these crystals starts to crystallize, it has been proposed that changes in solvent dynamics play a role in the observed temperature dependence of specific damage. However, only the results presented in the current paper clearly demonstrate that solvent in flash-cooled protein crystals is indeed liquid-like at its crystallization temperature. The decreased viscosity can influence specific radiation damage in two ways; either through increased radical mobility or *via* increased protein flexibility if one assumes protein and solvent dynamics to be coupled (Fenimore *et al.*, 2002). The unit-cell volume of a protein crystal

increases linearly with dose at 100 K (Ravelli & McSweeney, 2000), which might be due to charge repulsion of the created radicals (Ravelli *et al.*, 2002). Supportive evidence for a decreased viscosity of the solvent at its crystallization temperature comes from nonlinear cell volume increase (Weik, Ravelli *et al.*, 2001) or even a decrease in cell volume (Ravelli *et al.*, 2002), which has been attributed to radicals being able to recombine owing to increased rotational and translational freedom. By thawing and re-flash-cooling irradiated crystals of cyctochrome P450cam, it has been suggested that solvated electrons become transiently mobile and generate an intermediate state in the enzyme (Schlichting *et al.*, 2000). The observation of liquid-like solvent at the crystallization temperature allows the rational design of temperature-controlled crystallographic studies that aim to identify specific radiation-damage features that cannot be observed at 100 K because either certain radicals are not mobile or the protein is not flexible enough. As regards the trigonal trypsin crystals, we have collected data sets at 100, 130, 160 and 200 K and could observe a change in radiation sensitivity and characteristics at 200 K (results to be published elsewhere). Studying the temperature dependence of specific radiation damage is pertinent for the following reasons: (*a*) X-ray beam heating of the crystal to temperatures above 100 K might occur when heavily absorbing elements are present, such as Fe atoms in ferritin (Ravelli *et al.*, 2002), even if the cold stream operates at 100 K; (*b*) crystallographic data are sometimes collected at temperatures higher than 100 K if an enzymatic intermediate state is to be accumulated; (*c*) new radiation damage features can be observed; and (*d*) specific radiation damage can be employed as a marker for temperature-dependent protein dynamics. The identification of temperature windows in which the crystal solvent is liquid-like may serve as a basis for designing kinetic crystallography experiments, which aim to examine and exploit the coupling between protein and solvent dynamics to accumulate and trap enzymatic intermediate states.

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